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ANIMAL BIOTECHNOLOGY

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SYLLABI-BOOK MAPPING TABLE

Animal Biotechnology

Syllabi	Mapping in Book
BLOCK-I GENETIC ENGINEERING **UNIT-1 OVERVIEW OF ANIMAL BIOTECHNOLOGY** Overview of Animal Biotechnology, Basic Principles of Genetic Engineering, Genetic Engineering in Animal Systems.	**Unit 1:** Overview of Animal Biotechnology **(Pages 1-18)**
UNIT -2 VECTORS: PLASMID, COSMID, AND PHAGEMIDS Vectors: Plasmid, Cosmid, Phagemids - Yeast Artificial Chromosome (YAC) and Bacterial Artificial Chromosome (BAC), Shuttle Vectors, Yeast Vectors, Minichromosomes.	**Unit 2:** Vectors: Plasmid, Cosmid, and Phagemids **(Pages 19-52)**
UNIT-3 GENE TRANSFER METHODS IN ANIMALS Gene Transfer Methods in Animals, Electroporation, Microinjection, Biolistic Particle Delivery Systems, Sonoporation, Optical Transfection, Protoplast Fusion, Retrovirus Mediated Gene Transfer.	**Unit 3:** Gene Transfer Methods in Animals **(Pages 53-66)**
BLOCK-II ANIMAL CELL CULTURE AND TRANSGENIC ANIMALS **UNIT-4 CONSTITUENTS OF CULTURE MEDIA** Media Requirements, Preparation of Media and Sterilization Techniques, Natural and Synthetics Media, Culture Methods, Hanging Drop, Suspension and Monolayer Culture, Primary and Established Cell Lines.	**Unit 4:** Constituents of Culture Media **(Pages 67-84)**
UNIT-5 CHARACTERISTICS OF TRANSFORMED CELLS Characteristics of Transformed Cells, Methods of Cell Preservations, Applications of Cell Culture in Product Development and Tissue Repair, Bioreactors and Scaling-Up Technologies.	**Unit 5:** Characteristics of Transformed Cells **(Pages 85-100)**
UNIT-6 PRODUCTION AND APPLICATIONS OF TRANSGENIC ANIMALS Production and Applications of Transgenic Animals (Fish, Mice, Sheep and Pig) and Chimeras, Dolly.	**Unit 6:** Production and Applications of Transgenic Animals **(Pages 101-114)**
UNIT-7 TRANSGENIC ANIMALS FROM FOETAL CELLS Transgenic Animals from Foetal Cells, Transgenic Animals in Xenotransplantation, Transgenic Organisms to Interrupt Disease Cycles, Artificial Insemination and Embryo Transfer.	**Unit 7:** Transgenic Animals from Foetal Cells **(Pages 115-126)**

BLOCK-III MOLECULAR DIAGNOSTIC TECHNIQUES AND PHEROMONES

UNIT-8 ISOLATION AND PURIFICATION OF NUCLEIC ACIDS

Isolation and Purification of Nucleic Acids, Hybridization: Southern, Western and Northern Hybridization.

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UNIT-11 STEM CELLS

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UNIT-12 RECOMBINANT VACCINES

Recombinant Vaccines, Production of Insulin and Tissue Plasminogen Activator.

Recombinant Vaccines, Subunit Vaccines and Live Vaccines - Production of Insulin and Tissue Plasminogen Activator.

UNIT-13 GENE THERAPY

Gene Therapy, Somatic Gene Therapy, Germline Gene Therapy, Applications of Advanced Techniques, Positron Emission Tomography-Computed Tomography (PET-CT), Molecular Imaging, Human Genome Project.

UNIT-14 GENETIC ENGINEERING - TRANSGENICS

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INTRODUCTION

NOTES

Animal biotechnology is the combination of science and engineering which is very useful to transform living organisms. The main objective of animal biotechnology is to make products, to improve animals, and to develop microorganisms for specific agricultural uses. Creating transgenic animals is the best example of animal biotechnology.

Biotechnology is the use of artificial methods to modify the genetic material of living organisms or cells to produce novel compounds or to perform new functions. Biotechnology has been used for improving livestock and crops since the beginning of agriculture through selective breeding. Since the discovery of the structure of DNA (1953) and principally after the development of methods to manipulate DNA (1970), biotechnology is equally significant to manipulate organisms' DNA at the molecular level.

This technology has key applications in medicine for the production of Vaccines and Antibiotics, and in agriculture for the genetic modification of crops. In addition, biotechnology has many industrial applications, such as fermentation, the treatment of oil spills, and the production of biofuels, as well as many household applications, such as the use of Enzymes in laundry detergent.

Genetic engineering, also called genetic modification or genetic manipulation, is the direct manipulation of an organism's genes using biotechnology. It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms. New DNA is obtained by either isolating or copying the genetic material of interest using recombinant DNA methods or by artificially synthesising the DNA. A construct is usually created and used to insert this DNA into the host organism. The first recombinant DNA molecule was made by Paul Berg in 1972 by combining DNA from the monkey virus SV40 with the lambda virus. As well as inserting genes, the process can be used to remove, or "Knock Out", genes. The new DNA can be inserted randomly, or targeted to a specific part of the genome.

Gene therapy is a medical field which focuses on the genetic modification of cells to produce a therapeutic effect or the treatment of disease by repairing or reconstructing defective genetic material. The first attempt at modifying human DNA was performed in 1980 by Martin Cline, but the first successful nuclear gene transfer in humans, approved by the National Institutes of Health, was performed in May 1989. The first therapeutic use of gene transfer as well as the first direct insertion of human DNA into the nuclear genome was performed by French Anderson in a trial starting in September 1990. It is thought to be able to cure many genetic disorders or treat them over time.

This book, *Animal Biotechnology*, is divided into four blocks, which are further subdivided into fourteen units. The topics discussed include animal

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biotechnology, basic principles of genetic engineering, genetic engineering in animal systems, vectors: plasmid, cosmid, and phagemids, Yeast Artificial Chromosome (YAC) and Bacterial Artificial Chromosome (BAC), shuttle vectors, yeast vectors, minichromosomes, gene transfer methods in animals –electroporation, microinjection, biolistic particle delivery system, and sonoporation, preparation of media and sterilization techniques, natural and synthetic media, culture methods, hanging drop, characteristics of transformed cells, bioreactors and scaling-up technologies, production and applications of transgenic animals, transgenic animals from foetal cells, transgenic animals in xenotransplatation, artificial insemination and embryo transfer, isolation and purification of nucleic acids, hybridization: Southern, Western and Northern hybridization, PCR, RFLP, RAPD, DNA finger-printing, DNA bar coding, Maxam and Gilbert method, Sanger's di-deoxy method and automated DNA sequencing, pheromones, knock out and knock in technology, gene therapy, human genome project, and ethical issues in genetic engineering and transgenics.

The book follows the Self-Instructional Mode (SIM) wherein each unit begins with an 'Introduction' to the topic. The 'Objectives' are then outlined before going on to the presentation of the detailed content in a simple and structured format. 'Check Your Progress' questions are provided at regular intervals to test the student's understanding of the subject. 'Answers to Check Your Progress Questions', a 'Summary', a list of 'Key Words', and a set of 'Self-Assessment Questions and Exercises' are provided at the end of each unit for effective recapitulation.

BLOCK - I
GENETIC ENGINEERING

NOTES

UNIT 1 OVERVIEW OF ANIMAL BIOTECHNOLOGY

Structure

1.0 INTRODUCTION

Biotechnology is the use of artificial methods to modify the genetic material of living organisms or cells to produce novel compounds or to perform new functions. Biotechnology has been used for improving livestock and crops since the beginning of agriculture through selective breeding. Since the discovery of the structure of DNA (1953) and principally after the development of methods to manipulate DNA (1970), biotechnology is equally significant to manipulate organisms' DNA at the molecular level. This technology has key applications in medicine for the production of Vaccines and Antibiotics, and in agriculture for the genetic modification of crops. In addition, biotechnology has many industrial applications, such as fermentation, the treatment of oil spills, and the production of biofuels, as well as many household applications, such as the use of Enzymes in laundry detergent.

Animal biotechnology is the combination of science and engineering which is very useful to transform living organisms. The main objective of animal biotechnology is to make products, to improve animals, and to develop microorganisms for specific agricultural uses. Creating transgenic animals is the best example of animal biotechnology.

The basic technique is based on nucleic acids which are macromolecules made of nucleotides - a sugar, a phosphate and a nitrogenous base. The phosphate groups on these molecules each have a net negative charge. An entire set of DNA molecules in the nucleus of Eukaryotic organisms is called the Genome. DNA has two complementary strands linked by hydrogen bonds between the paired bases.

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Unlike DNA in Eukaryotic cells, RNA molecules leave the nucleus. Messenger RNA (mRNA) is analysed most frequently because it represents the Protein-Coding Genes that are being expressed in the cell.

In this unit, you will study about the overview of animal biotechnology, basic principles of genetic engineering, and genetic engineering in animal systems.

1.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the overview of animal biotechnology
- Define the basic principles of genetic engineering
- Analyse the genetic engineering in animal systems

1.2 OVERVIEW OF ANIMAL BIOTECHNOLOGY

Biotechnology, commonly abbreviated as biotech, is the broad area of biology involving living systems and organisms to develop or make products, or 'Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use'.

Depending on the tools and applications, it often overlaps with the related fields of molecular biology, bio-engineering, biomedical engineering, bio-manufacturing, molecular engineering, etc.

Biotechnology is technology that utilizes biological systems, living organisms or parts of this to develop or create different products. Brewing and Baking Bread are examples of processes that fall within the concept of biotechnology, such as use of Yeast to produce the desired product. Such traditional processes usually utilize the living organisms in their natural form (or further developed by breeding), while the more modern form of biotechnology will generally involve a more advanced modification of the biological system or organism.

The biotechnology simply can be defined as technology based on biology. The term biotechnology includes cellular and biomolecular processes to develop technologies and products that assist and advance our lives and health. The applications of biological processes of microorganisms for more than 6,000 years make useful food products, such as Bread and Cheese, and to preserve dairy products. Modern biotechnology provides cutting edge products and technologies to combat debilitating and rare diseases, reduce our environmental footprint, feed the hungry, and use less and cleaner energy, and have safer, cleaner and more efficient industrial manufacturing processes. At present, there are more than 250 Biotechnology Health Care Products and Vaccines available to patients, largely for previously untreatable diseases. More than 13.3 million farmers round the world using agricultural biotechnology to raise yields, reduce damage from pests and farming's impact on environment. The recent trends and advances in

biotechnology are helping us get ready for and meet society's most imperative challenges.

The term biotechnology was coined by a Hungarian Engineer, Karl Ereky (1917), to describe a process for large scale production of Pigs. According to him every jobs are biotechnology by which products are produced from raw materials using living organisms. At the conclusion of 20th century, biotechnology emerged as a new dimension of biology amalgamated with technology. There was no rapid sprout of this discipline, but some methods were developed centuries back. Therefore, in nut shell, biotechnology is concerned with utilization of biological components for creation of useful products.

Thus biotechnology has been broadly defined as, 'The development and employment of biological processes, forms and systems to obtain greatest benefits to man and other forms of life'.

It can also be defined as, 'The science of applied biological process'.

The integrated use of biochemistry microbiology and engineering sciences in order to achieve technological application of the capabilities of microorganisms, cultured tissues, cells, and parts their off termed biotechnology.

History of Biotechnology

The origin of biotechnology is as ancient as human civilization. The development of biotechnology can be defined considering its growth in following two phases:

(i) Classical Biotechnology

(ii) Advanced Biotechnology

Classical Biotechnology: The classical or traditional biotechnology is the technology which was developed by our ancestors using the fermentation of Bacteria, such as the animal fat 'Ghee' which is a fermented product of milk. Similarly, the preparations of Curd, Wine, Beer, vinegar, etc., all are the fermented products prepared using biotechnology. In Indian Ayurveda, the production of 'Asava' and 'Arista' is done using different substrates and flowers of Mahua (*Madhuca indica)* or Dhataki (*Wodfordia fructicosa)* which is characterized till today since Vedic period. In all these methods, various substrates are transformed into a number of products.

Advanced Biotechnology: The two key features that differentiates the advanced biotechnology or modern biotechnology from traditional biotechnology are as follows:

(i) Capability of science to change genetic material for getting new products for specific requirement through Recombinant DNA Technology or RDT.

(ii) Possession or ownership of technology and its socio-political impact.

At the moment conventional industries, pharmaceutical industries, agro-industries, etc., are targeting their attention to obtain biotechnology-based products. The new or modern biotechnology embraces methods of genetic modification and cell fusion, and fermentation technology to find progressively more and wide industrial applications.

Biotechnology as Interdisciplinary Science

The key difference between biology and biotechnology is their scale of operation. Usually biologist works in the range of nanograms to milligrams, however biotechnologists working on production of vaccine may be satisfied with milligram yields, but many other projects aims at kilograms or tones. Thus, the main objective of biotechnologists consists of scaling-up the biological processes (Smith, 1996) for large scale cultivation of microbes and cells, their upstream and downstream process, etc. These processes have been separated into five major operations, as follows:

(i) Strain Selection and Improvement

(ii) Mass Culture

(iii) Optimization of Cell Responses

(iv) Process Operation

(v) Downstream Processing (Product Recovery)

Many areas of biotechnology have arisen through the interaction between various parts of biology and engineering, biochemistry, biophysics, cell biology, colloid chemistry, embryology, ecology, genetics, immunology, molecular biology, medical chemistry, pharmacology, polymer chemistry, thermo-chemistry, and virology. The modern biotechnology has developed several technologies but always extracting basic knowledge from biology. Figure 1.1 illustrates the interdisciplinary nature of biotechnology.

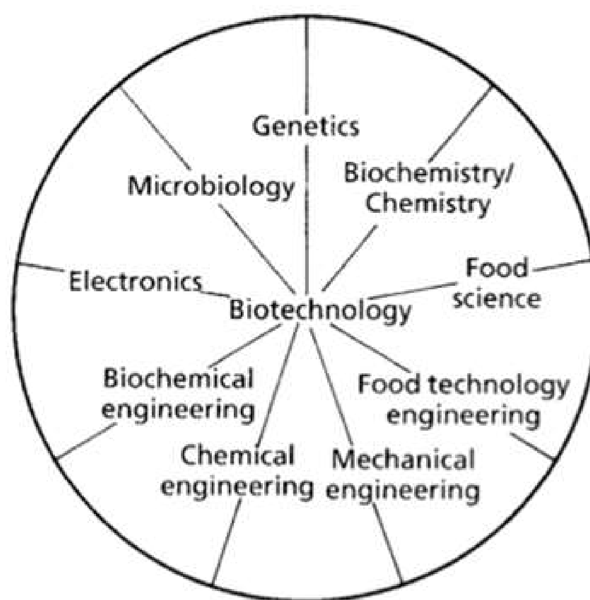


Fig. 1.1 *The Interdisciplinary Nature of Biotechnology*

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Biotechnology in India

After the global application of rDNA technology, the Government of India has set up an official agency, the National BioTechnology Board (NBTB) which started functioning under the Department of Science and Technology (DST) in 1986. NBTB was replaced with a full-fledged department, the Department of Biotechnology (DBT), under the ministry of Science and Technology for planning, promotion and coordination of various programs. Today, India has the DBT, DST, CSIR, ICAR, ICMR, ICGEB and IARI, and other agencies which are working under the Government. These agencies and the other National and International Industries are manufacturing Biotech products and marketing them after clinical trials. A Technology Development Board (TDB) has been set up by the Government for the promotion of product that are developed and tested. The Technology Information, Forecasting and Assessment Council (TIFAC) have prepared a 'Vision 2020' document which consists of biotechnology also. Since 1980s, India has supported a lot to biotechnology industry and its products so that currently there are over 30 companies in India which are producing modern biotech products. Figure 1.2 illustrates the schematic outline of biotechnology, genetic engineering and its various applications.

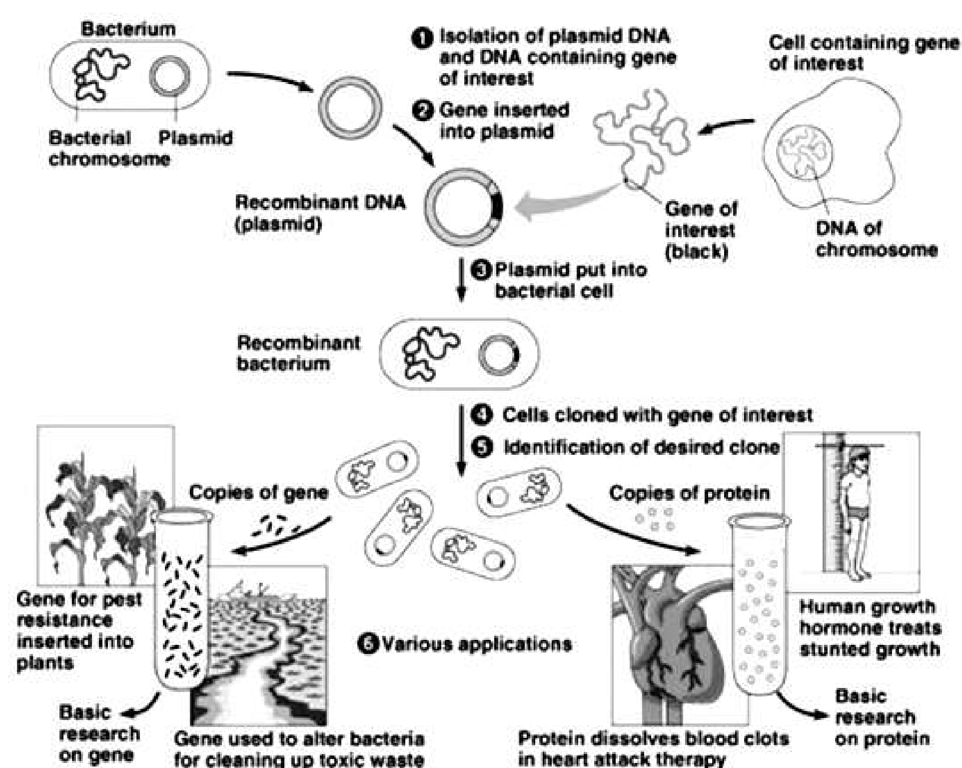


Fig. 1.2 *Schematic Outline of Biotechnology, Genetic Engineering and its Applications*

Humans have altered the genomes of species for thousands of years through selective breeding, or artificial selection as contrasted with natural selection. More recently, mutation breeding has used exposure to chemicals or radiation to produce a high frequency of random mutations, for selective breeding purposes. Genetic engineering is the direct manipulation of DNA by humans outside of their original location. Watson and Crick (1953) showed that DNA molecule has a double-helix structure. A unified definition of 'Genetic Engineering' has been given by Smith (1996) as, 'The formation of new combinations of heritable material by the insertion of nucleic acid molecules produced by whatever means outside the cell into any Virus, Bacterial Plasmid or other Vector System so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation'.

Until the early 1970s DNA was the most difficult cellular molecule for the biochemist to analyze. Enormously long and chemically monotonous, the string of nucleotides that forms the genetic material of an organism could be examined only indirectly, by Protein or RNA sequencing or by genetic analysis. But with the advancement of technology, the macromolecule of cell DNA can be easily analyzed. It is now possible to isolate a specific region of a genome to produce a virtually unlimited number of copies of it, and to determine the sequence of its nucleotides. At the height of invention, automated machines are generating DNA sequences at the rate of 1000 nucleotides per second, round the clock. By related techniques, an isolated gene can be altered (engineered) and transferred back into germ line of an animal or plant, so as to become a functional and heritable part of organism's genome.

In 1978, a U.S. Company 'Genetech' used genetic engineering technique to produce human insulin in *Escherichia coli*. In 1996, First Clone Lamb 'Dolly' was borne successfully by the efforts of scientists of Scotland. Thereafter several cloned animals were produced in 2001, and sequence of Human Genome was completed by March 2003. On December 27, 2002, a claim was made for birth of a Clone Baby 'Eve' by scientists of 'Human Cloning Society, the Clonaid' of France. In May 2005, scientists in South Korea have used a method called therapeutic cloning to produce stem cell lines. In this method, human embryos were produced through cloning (as done for Dolly) and stems cells were obtained from blastocyst. The excised stem cells could be grown in-vitro and used after.

Bioinformatics is a hybrid science that links biological data with techniques for information storage, distribution, and analysis to support multiple areas of scientific research including biomedicine. Bioinformatics is fed by high-throughput data-generating experiments, including genomic sequence determinations and measurements of gene expression patterns. Database projects curate and annotate the data and then distribute it via the World Wide Web (WWW). Mining these data leads to scientific discoveries and to the identification of new clinical

applications. In the field of medicine in particular, a number of important applications for bioinformatics have been discovered. For example, it is used to identify correlations between Gene Sequences and Diseases, to predict Protein Structures from Amino Acid Sequences, to aid in the design of novel drugs, and to tailor treatments to individual patients based on their DNA Sequences (Pharmacogenomics).

Applications of Biotechnology

The modern biotechnology includes the scientific methods, such as genetic engineering have enhanced the natural capabilities of natural production of organisms. Bacteria like *Escherichia coli* are producing Mammalian Hormones, such as Insulin, Somatostatin, Somatotropin, etc. Yeast cells have been genetically manipulated to produce Vaccine against Hepatitis B Virus (Hepatitis Disease), Myeloma Cells (Cancerous Cells of Bone Marrow) and B-Cells of immunized Mice were hybridized to produce Hybrid Cells that consisted characteristics of both the cells which were cell division and antibody production. Now the Hybrid Cells (Hybridomas) are being used for the production of Monoclonal Antibodies. Following are some of the areas where biotechnology has significant role.

1. The maximum benefits of biotechnology have been utilized by health care. Biotechnology derived Proteins and Polypeptides form the new class of potential drugs like Humulin (Human Insulin), Recombivax HB (Hepatitis B Vaccines), etc.
2. Biotechnology is making new ground in food and agriculture area. Food biotechnology provides valuable and viable alternatives to food problems and a solution to nutritionally influenced diseases, such as Diabetes, Arthritis, etc.
3. The major landmark in human history is the production of Human Genome Sequence. The Human Genome Project (HGP) is an international research program. Almost the whole human genome has been sequenced and chromosome map has been developed in various laboratories world-wide through coordinated efforts. Human chromosome mapping was completed by March 2, 2003, and about 33,000 functional genes in human. More than 97% genes are non-functional. They do not encode for any polypeptide chain. Objectives of human genome project are to construct the detail genetic and physical map of human genome, to determine the complete nucleotide sequence of human DNA, to store information in database, to locate the estimated 50,000-10,000 genes within the human genome, to address the Ethical, Legal and Social Issues (ELSI) that may arise from the project, and to perform similar analysis on the genomes of several other organisms.
4. The natural biodegradability of pollutants present in environment has increased with the use of biotechnology. The bioremediation technologies have been

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found successful to combat the pollution problems. Bioremediation is the use of microorganisms to detoxify pollutants, present in the environment usually as soil or water sediments. The pollutants cause several health problems. Microorganisms which show potential to degradation of oil, pesticides and fertilizers belong to the genera of Bacteria, such as *Pseudomonas, Micrococcus, Bacillus*, and Fungi, such as *Candida, Cladosporium, Torulopsis, Trichoderma,* etc.

5. Computer-based study and designing of 'Genome' is called 'Genomics'. Genomics deals with sequencing of the complete genome of a particular organism. Similarly, study of Proteins present on Genome using computer is called Proteomics. The Proteomics can be defined as the study of all the Proteins present in the Genome of an organism. With the help of Proteomics and Genomics the new molecules that can interact with the other partners could be identified. This gives us deep insight into biological pathway.
6. The bioinformatics is a new field of biotechnology linked with information technology. Bioinformatics may be defined as application of Information Sciences (Mathematics, Statistics, and Computer Sciences) to increase the understanding of Biology, Biochemistry and Biological Data. The most remarkable success of bioinformatics to date has been its use in the shotgun Sequencing of Human Genome.

1.3 BASIC PRINCIPLES OF GENETIC ENGINEERING

The Bacteria and other Prokaryotes comprising host controlled Restriction Modification (RM) system is found in organisms, and aids a mechanism of defense against foreign DNA, borne by Bacteriophages. These organisms have Restriction Enzymes, also called Restriction Endonucleases or REs, which cut dsDNA at specific points into fragments, which are then degraded further by other endonucleases. About one-fourth of known Bacteria acquire RM systems and of those about one-half have more than one type of system. The sequences recognized by the restriction enzymes are very short. The presence of Methyl groups helps in order to prevent destruction of its own DNA by the restriction enzymes. The endonucleases cleave internal/non-terminal phosphodiester bonds. Restriction endonucleases cleave internal phosphodiester bonds only after recognizing specific sequences in DNA which are usually 4-6 base pairs long, and often palindromic.

Nuclease is a type of enzyme that has capacity to cleave nucleic acids. The nucleases are mostly definite in action, thus ribonucleases acting only upon RiboNucleic Acids (**RNA**) and deoxyribonucleases upon DeoxyriboNucleic Acids (**DNA**). Some enzymes having a general action, for example Phosphoesterases can be called Nucleases and are found in both Flora and

Fauna. There are two major types of nucleases based on their site of action, namely the Exonucleases and Endonucleases. Exonucleases are competent of removing nucleotides one at a time from a DNA molecule whereas endonucleases work by cleaving the phosphodiester bonds within DNA molecule. The restriction enzymes are nucleases that split only those DNA molecules in which they recognize particular subunits. There are four types of restriction enzymes are recognized on the basis of their structure, site specificity and cofactors. Thus some RE split the target DNA molecule at random sites (Type I), but others split the molecule only at the recognition site (Type II) or at a fixed distance from the recognition site (Type III). Type II and Type III restriction enzymes are powerful tools in the elucidation of the sequence of bases in DNA molecules. Type IV restriction enzymes cleave only methylated DNA with weak sequence specificity. Restriction Enzymes (RE) play a fundamental role in recombinant DNA (rDNA) technology or Genetic Engineering (GE).

The recognition sequences in DNA differ for each restriction enzyme, producing differences in size, sequence and orientation of a sticky end (5´/3´end) 'Overhang' of an enzyme restriction. The ***Eco*RI** digestion produces sticky ends, as follows.



Whereas, ***Sma*I** restriction enzyme produces blunt ends, as follows.



There are different enzymes used in DNA manipulation, one of them is nuclease, and others are DNA polymerases, kinases, alkaline phosphatases, and topoisomerases. The nuclease have crucial roles in various DNA repair systems, which involve DNA replication, base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair. The structural and mechanical deformities of these nucleases may leads in genetic instability or severe immunodeficiency. Thus, structural biology and 3D structural information of nucleases are providing important insights into molecular architectures, DNA recognition and cleaving mechanism.

Invention of Host Controlled Restriction Modification

The Restriction Modification (RM) system was initially discovered by S. Luria and M. Human (1952-1953). They noticed that Bacteriophage growing within an Infected Bacterium could be modified by the release and re-infection of a related Bacterium, the Bacteriophage's. Thus RM system is found in Bacteria and other Prokaryotes, and assists in defense against foreign DNA, that may borne by

Bacteriophages. Later on J. Weigle and G. Bertani (1953) and subsequently many other scientists led to understanding that restriction was due to attack and breakdown of modified bacteriophage's DNA by specific enzymes of Recipient Bacteria. Further H.O. Smith isolated ***Hin*DII**, the first known Restriction Enzyme (RE), can be used for restriction mapping. The extensive work and isolation of these enzymes in laboratory gave a path for controlled manipulation of DNA, and thus providing foundation for development of genetic engineering. In 1978, W. Arber, D. Nathans, and H.O. Smith were awarded the Nobel Prize in Physiology for Medicine for their outstanding contribution on restriction-modification. Figure 1.3 illustrates the Restriction Modification or RM system control Horizontal Gene Transfer.

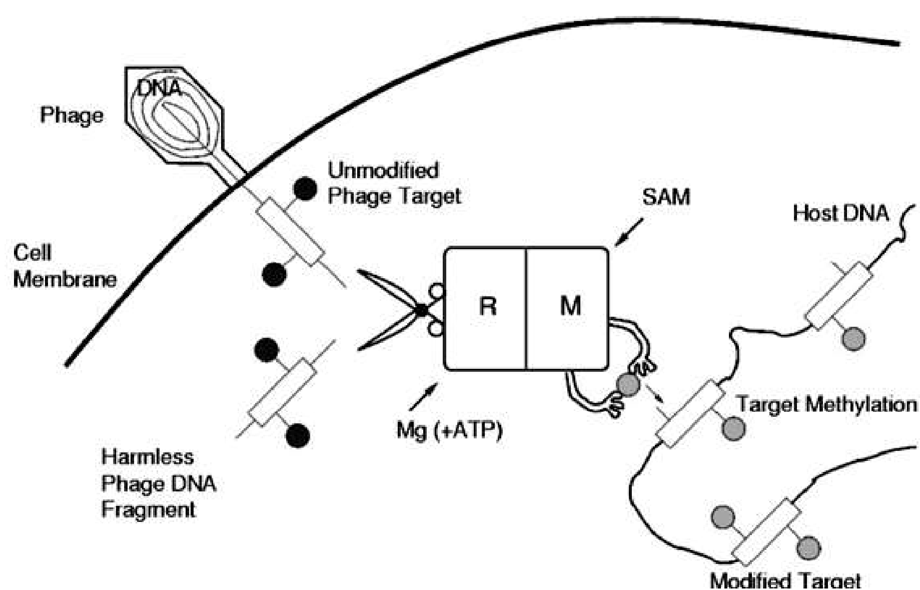


Fig. 1.3 *Restriction Modification (RM) System Control Horizontal Gene Transfer*

The host controlled restriction and modification are most readily observed when bacteriophages are transferred from one bacterial host strain to another. For the stock grounding of phage λ upon *Escherichia coli,* the strain C and strain K were used in experiments. The phages are said to be restricted by second host strain *Escherichia coli* K. When those phages that do result from the infection of *Escherichia coli* K are now replated on *Escherichia coli* K they are no longer restricted, but if they are first cycled through *Escherichia coli* C they are once again restricted when plated upon *Escherichia coli* K. Thus the efficiency with which phage plates upon a particular host strain depends upon the strain on which it was last propagated. This non-heritable change in strain without further restriction is called modification. The restricted phages adsorb to restrictive hosts and inject their DNA normally. When the phage are labeled with 32P it is apparent that their

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DNA is degraded soon after injection and the endonuclease that is primarily responsible for this degradation is called a restriction endonuclease or restriction enzyme. The restrictive host must of course protect its own DNA from the potentially lethal effects of the restriction endonuclease and so its DNA must be appropriately modified. Figure 1.4 illustrates the host controlled restriction and modification of Phage λ in *Escherichia coli* analyzed by Efficiency Of Plating (E.O.P.).

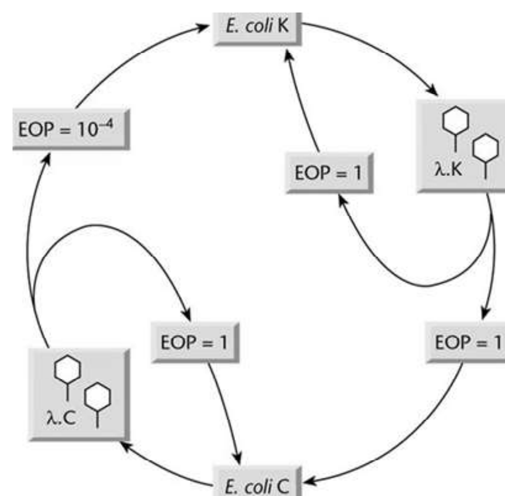


Fig. 1.4 *Host Controlled Restriction and Modification of Phage λ in Escherichia coli Analyzed by Efficiency Of Plating (E.O.P.)*

The modification involves Methylation of certain bases at a very limited number of sequences within DNA which constitute the recognition sequences for the restriction endonuclease. Although phage infections have been chosen as the example to illustrate restriction and modification, these processes can occur whenever DNA is transferred from one bacterial strain to another. The conjugation, transduction, transformation and transfection are also subject to host controlled restriction. The genes that identify host controlled restriction and modification systems may be inherent upon host chromosome itself or may be located on a Plasmid or Prophase 1 (P1).

Types of Restriction Modification

There are four categories of restriction modification systems, namely the Type I, Type II, Type III and Type IV, all with RE and Methylase Activity (MA) except for Type IV (that has no MA). They were coined in sequence of discovery, although Type II system is the most common in biotechnology.

Type I Systems: Type I are the most complex restriction modification system, consisting of 3 polypeptides, such as R (Restriction), M (Modification), and S (Specificity). The resulting complex can cleave and methylate DNA as well, require ATP, and cleavage often occurs at considerable distance from recognition site.

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Type II Systems: The Type II restriction modification systems are the simplest and most prevalent systems. Instead of working as a complex, the Methyltransferase and Endonuclease are encoded as two separate Proteins and act independently because of the absence of specificity Protein. The Methyltransferase are monomer, methylating a strand at a time. The Endonuclease works as homodimer and facilitates cleavage of both strands at a defined position close to or within the recognition sequence. Therefore, Type II systems are used in labs for DNA Analysis and Gene Cloning.

Type III Systems: The Type III restriction modification systems have R (Res) and M (Mod) Proteins that form a complex of modification and cleavage. Methylation only occurs on one strand of DNA unlike most other known mechanisms. The heterodimer formed by R and M Proteins competes with itself by modifying and restricting the same reaction.

Type IV Systems: The Type IV restriction modification systems are not true RM systems because they only contain a restriction enzyme. Contrasting other Type IV restriction enzymes recognize and cut only modified DNA.

Functions of Restriction and Modification System

Following are the functions of restriction and modification system:

- The *Neisseria meningitides* has multiple Type II restriction endonuclease systems that are employed in natural genetic transformation process by which a recipient bacterial cell can take up DNA from a neighboring donor bacterial cell and integrate this DNA into its genome by recombination.
- Restriction modification appears to be a major driver of sexual isolation and speciation in the Meningococci.
- Restriction modification systems can also act as selfish genetic elements, forcing their maintenance on the cell through post-segregational cell killing.
- Some Viruses have evolved ways of subverting the restriction modification system, usually by modifying their own DNA, by adding Methyl or Glycosyl groups to it, thus blocking the restriction enzymes. Other Viruses, such as Bacteriophages T3 and T7, encode Proteins that inhibit the restriction enzymes.
- Many Prokaryotes have developed multiple types of restriction modification systems.

Applications of Restriction Modification System

Molecular Biology: Restriction modification systems can be cloned into plasmids and screened because of resistance provided by methylation enzyme. Restriction enzymes are also used to analyze composition of DNA in reference to presence or absence of mutations that may affect specificity of REase cleavage specificity. When wild-type and mutants are analysed by digestion with different REases, the gel electrophoretic products vary in length, largely because mutant genes RFLP (Restriction Fragment Length Polymorphism) will not be cleaved in a

similar pattern as wild-type that provide the REases nonspecific to mutant sequence.

Gene Therapy: The bacteria RM system has been proposed as a model for devising human anti-viral gene or genomic vaccines and therapies in view of the fact that RM system serves an innate defense-role in bacteria by restricting tropism of bacteriophages. Researches on REases and Zinc Finger Nucleases (ZFN) show that it can cleave DNA of various human Viruses (HSV-2 and HIV-1), with the ultimate goal of inducing target mutagenesis and aberrations of human infecting Viruses. The ZFNs are a powerful tool for host genome editing due to their enhanced sequence specificity. Figure 1.5 illustrates the Zinc Finger Nucleases (ZFNs) which are highly specific genomic scissors.

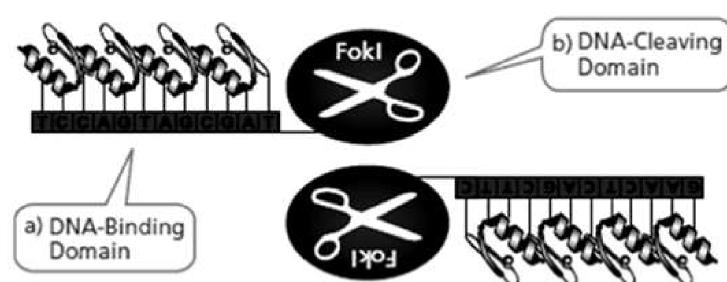


Fig. 1.5 *Zinc Finger Nucleases: Highly Specific Genomic Scissors*

Mobile Genetic Elements (MGEs): RM systems are key players in co-evolutionary interaction between Mobile Genetic Elements (MGEs) and their hosts. The genes in plasmids, prophages, transposons, Integrative Conjugative Elements (ICEs) and integrons are MGEs that can move between Prokaryotic Genome by the utilization of natural transformation mechanism, vesicles, nanotubes, and the gene transfer agents.

1.4 GENETIC ENGINEERING IN ANIMAL SYSTEMS

Genetic engineering, also called genetic modification or genetic manipulation, is the direct manipulation of an organism's genes using biotechnology. It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms. New DNA is obtained by either isolating or copying the genetic material of interest using recombinant DNA methods or by artificially synthesising the DNA. A construct is usually created and used to insert this DNA into the host organism. The first recombinant DNA molecule was made by Paul Berg in 1972 by combining DNA from the monkey virus SV40 with the lambda virus. As well as inserting genes, the process can be used to remove, or "Knock Out", genes. The new DNA can be inserted randomly, or targeted to a specific part of the genome.

An organism that is generated through genetic engineering is considered to be Genetically Modified (GM) and the resulting entity is a Genetically Modified Organism (GMO). The first GMO was a bacterium generated by Herbert Boyer and Stanley Cohen in 1973. Rudolf Jaenisch created the first GM animal when he inserted foreign DNA into a mouse in 1974. The first company to focus on genetic engineering, Genentech, was founded in 1976 and started the production of human proteins. Genetically engineered human insulin was produced in 1978 and insulin-producing bacteria were commercialised in 1982. Genetically modified food has been sold since 1994, with the release of the Flavr Savr tomato. The Flavr Savr was engineered to have a longer shelf life, but most current GM crops are modified to increase resistance to insects and herbicides. GloFish, the first GMO designed as a pet, was sold in the United States in December 2003. In 2016 salmon modified with a growth hormone were sold.

Genetic engineering has been applied in numerous fields including research, medicine, industrial biotechnology and agriculture. In research GMOs are used to study gene function and expression through loss of function, gain of function, tracking and expression experiments. By knocking out genes responsible for certain conditions it is possible to create animal model organisms of human diseases. As well as producing hormones, vaccines and other drugs, genetic engineering has the potential to cure genetic diseases through gene therapy. The same techniques that are used to produce drugs can also have industrial applications such as producing enzymes for laundry detergent, cheeses and other products.

Genetic engineering is an important tool for natural scientists, with the creation of transgenic organisms one of the most important tools for analysis of gene function. Genes and other genetic information from a wide range of organisms can be inserted into bacteria for storage and modification, creating genetically modified bacteria in the process. Bacteria are cheap, easy to grow, clonal, multiply quickly, relatively easy to transform and can be stored at -80 °C almost indefinitely. Once a gene is isolated it can be stored inside the bacteria providing an unlimited supply for research. Organisms are genetically engineered to discover the functions of certain genes. This could be the effect on the phenotype of the organism, where the gene is expressed or what other genes it interacts with. These experiments generally involve loss of function, gain of function, tracking and expression.

Animals or microorganisms that have been changed through genetic engineering are termed genetically modified organisms or GMOs. If genetic material from another species is added to the host, the resulting organism is called transgenic. If genetic material from the same species or a species that can naturally breed with the host is used the resulting organism is called cisgenic. If genetic engineering is used to remove genetic material from the target organism the resulting organism is termed a knockout organism.

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Check Your Progress

1. What is biotechnology?
2. Explain the animal biotechnology.
3. Define the term advanced biotechnology.
4. Explain the term genetic engineering in animal system.

1.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Biotechnology, commonly abbreviated as biotech, is the broad area of biology involving living systems and organisms to develop or make products, or 'Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use'. Biotechnology is technology that utilizes biological systems, living organisms or parts of this to develop or create different products.
2. Animal biotechnology is the combination of science and engineering which is very useful to transform living organisms. The main objective of animal biotechnology is to make products, to improve animals, and to develop microorganisms for specific agricultural uses. Creating transgenic animals is the best example of animal biotechnology.
3. Advanced biotechnology includes the two key features that differentiates the advanced biotechnology or modern biotechnology from traditional biotechnology which are, (i) Capability of science to change genetic material for getting new products for specific requirement through Recombinant DNA Technology or RDT, and (ii) Possession or ownership of technology and its socio-political impact.
4. Animals or microorganisms that have been changed through genetic engineering are termed genetically modified organisms or GMOs. If genetic material from another species is added to the host, the resulting organism is called transgenic.

1.6 SUMMARY

- Biotechnology, commonly abbreviated as biotech, is the broad area of biology involving living systems and organisms to develop or make products, or 'Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use'.

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- Biotechnology is technology that utilizes biological systems, living organisms or parts of this to develop or create different products. Brewing and baking bread are examples of processes that fall within the concept of Biotechnology, such as use of Yeast to produce the desired product.
- The term biotechnology was coined by a Hungarian Engineer, Karl Ereky (1917), to describe a process for large scale production of Pigs. According to him every jobs are biotechnology by which products are produced from raw materials using living organisms.
- Advanced biotechnology includes the two key features that differentiates the advanced biotechnology or modern biotechnology from traditional biotechnology which are, (i) Capability of science to change genetic material for getting new products for specific requirement through Recombinant DNA Technology or RDT, and (ii) Possession or ownership of technology and its socio-political impact.
- Animals or microorganisms that have been changed through genetic engineering are termed genetically modified organisms or GMOs. If genetic material from another species is added to the host, the resulting organism is called transgenic.

1.7 KEY WORDS

- **Biotechnology:** It has been broadly defined as, 'The development and employment of biological processes, forms and systems to obtain greatest benefits to man and other forms of life'.
- **Genetic engineering:** It is the direct manipulation of DNA by humans outside of their original location.
- **Animal biotechnology:** Animal biotechnology is the combination of science and engineering which is very useful to transform living organisms. The main objective of animal biotechnology is to make products, to improve animals, and to develop microorganisms for specific agricultural uses.
- **Advanced biotechnology:** Advanced biotechnology includes the two key features that differentiates the advanced biotechnology or modern biotechnology from traditional biotechnology which are, (i) Capability of science to change genetic material for getting new products for specific requirement through Recombinant DNA Technology or RDT, and (ii) Possession or ownership of technology and its socio-political impact.

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1.8 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. What do you understand by the biotechnology?
2. Elaborate on the animal biotechnology.
3. State the term advanced biotechnology.
4. Illustrate the genetic engineering in animal system.

Long-Answer Questions

1. Discuss briefly the animal biotechnology with the help of examples.
2. Explain the basic principles of genetic engineering.
3. Analyse the genetic engineering in animal systems. Give appropriate examples.

1.9 FURTHER READINGS

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UNIT 2 VECTORS: PLASMID, COSMID, AND PHAGEMIDS

NOTES

Structure

2.0 INTRODUCTION

The cloning vector is a small piece of DNA, taken out of a Virus, a bacteria or the cell of a higher organism, and can be stably maintained into a foreign DNA fragment for cloning purposes. The vector therefore contains characters that allow for convenient insertion or removal of a DNA fragment to or from vector. DNA fragments thus generated contain either Blunt ends or overhangs known as Sticky ends, and vector DNA and foreign DNA with compatible ends can then be joined together by molecular ligation.

A plasmid is a small, extra chromosomal DNA molecule within a cell that is physically separated from chromosomal DNA and can replicate independently. They are most commonly found as small circular, double-stranded DNA molecules in bacteria; however, plasmids are sometimes present in archaea and eukaryotic organisms. In nature, plasmids often carry genes that benefit the survival of the organism and confer selective advantage such as antibiotic resistance.

A cosmid is a type of hybrid plasmid that contains a Lambda phage cos sequence. They are often used as a cloning vector in genetic engineering. Cosmids can be used to build genomic libraries. They were first described by Collins and Hohn in 1978. Cosmids can contain 37 to 52 (normally 45) kb of DNA, limits based on the normal bacteriophage packaging size. They can replicate as plasmids if they have a suitable Origin of Replication (ORI): for example SV40 ori in mammalian cells, ColE1 ori for double-stranded DNA replication, or f1 ori for single-stranded DNA replication in prokaryotes. They frequently also contain a gene for selection such as antibiotic resistance, so that the transformed cells can be identified by plating on a medium containing the antibiotic. Those cells which did not take up the cosmid would be unable to grow.

A phagemid or phasmid is a DNA-based cloning vector, which has both bacteriophage and plasmid properties. These vectors carry, in addition to the origin

of plasmid replication, an origin of replication derived from bacteriophage. Unlike commonly used plasmids, phagemid vectors differ by having the ability to be packaged into the capsid of a bacteriophage, due to their having a genetic sequence that signals for packaging. Phagemids are used in a variety of biotechnology applications; for example, they can be used in a molecular biology technique called "Phage Display".

In this unit, you will study about the vectors: plasmid, cosmid, and phagemids, yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), shuttle vectors, yeast vectors, and minichromosomes.

2.1 OBJECTIVES

After going through this unit, you will be able to:

- Define the vectors: plasmid, cosmid, and phagemids
- Explain the yeast artificial chromosome (YAC)
- Illustrate the bacterial artificial chromosome (BAC)
- Elaborate on the shuttle vectors
- Interpret the yeast vectors
- Analyse the minichromosomes

2.2 VECTORS: PLASMID, COSMID, AND PHAGEMIDS

The **cloning vector** is a small piece of DNA, taken out of a Virus, a bacteria or the cell of a higher organism, and can be stably maintained into a foreign DNA fragment for cloning purposes. The vector therefore contains characters that allow for convenient insertion or removal of a DNA fragment to or from vector. DNA fragments thus generated contain either Blunt ends or overhangs known as Sticky ends, and vector DNA and foreign DNA with compatible ends can then be joined together by molecular ligation. After a DNA fragment has been cloned into a cloning vector, it may be further sub-cloned into another vector designed for more specific use. There are many types of cloning vectors, but the most commonly used ones are Genetically Engineered Plasmids. Cloning is generally first performed using *Escherichia coli*, and cloning vectors in *Escherichia coli* include Plasmids, Bacteriophages (such as, phage), Cosmids, and Bacterial Artificial Chromosomes (BACs). Some DNA, however, cannot be stably maintained in *Escherichia coli*, with very large DNA fragments, then Yeast may be used as cloning vectors and include Yeast Artificial Chromosomes (YACs). Thus artificial chromosomes are manufactured chromosomes in the context of Yeast Artificial Chromosomes (YACs), Bacterial Artificial Chromosomes (BACs), or Human Artificial Chromosomes (HACs). An artificial chromosome can carry a much larger DNA

fragment than other vectors. YACs and BACs can carry a DNA fragment up to 300,000 nucleotides long. Three structural necessities of an artificial chromosome include an origin of replication, a centromere, and telomeric end sequences.

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In recent years, a remarkable development has been made in manipulation of Prokaryotic and Eukaryotic DNA. You can isolate DNA from different sources and cut enzymatically at desired place and rejoin the DNA of two organisms at desired site. Similar to DNA enzymes, the other most important requirements for recombinant DNA technology is the cloning and expression vectors. The recombinant DNA is produced by cloning a foreign DNA isolated either from the Genome or Synthesised Chemically or as cDNA using mRNA molecule. However, cloning of this DNA can be done only when 'another DNA molecule' is available that may replicate in the transformed host cell. This 'other DNA molecule' used for joining the foreign DNA is called vectors. The vectors are the DNA molecules that can carry a foreign DNA segment and replicate inside the host cell. Vectors may be Plasmids, A Bacteriophage, Cosmids, Phagemids, Transposons, a Virus YAC or BAC.

In molecular biology, a vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed (e.g., Plasmid, Cosmid, Lambda (l) Phages). A vector containing foreign DNA is termed recombinant DNA. The four major types of vectors are Plasmids, Viral Vectors, Cosmids and Artificial Chromosomes. Of these, the most commonly used vectors are plasmids. Common to all engineered vectors are an origin of replication, a multicloning site, and a selectable marker. Thus vector itself is generally a DNA sequence that consists of an insert (transgene) and a larger sequence that serves as the 'Backbone' of the vector. The purpose of a vector which transfers genetic information to another cell is typically to isolate, multiply, or express the insert in the target cell. All vectors may be used for cloning and are therefore cloning vectors, but there are also vectors designed especially for cloning, while others may be designed specifically for other purposes, such as transcription and Protein expression. Vectors designed specifically for the expression of the transgene in the target cell are called expression vectors, and generally have a promoter sequence that drives expression of the transgene. Simpler vectors called transcription vectors are only capable of being transcribed but not translated: they can be replicated in a target cell but not expressed, unlike expression vectors. Transcription vectors are used to amplify their insert.

The manipulation of DNA is normally conducted on *Escherichia coli* vectors, which contain elements necessary for their maintenance in *Escherichia coli*. However, vectors may also have elements that allow them to be maintained in another organism, such as Yeast, plant or mammalian cells, and these vectors are called **shuttle vectors**. Such vectors have bacterial or viral elements which may be transferred to the non-bacterial host organism, however, other vectors termed intragenic vectors have also been developed to avoid the transfer of any genetic material from an Alien Species. Insertion of a vector into the target cell is usually

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called transformation for Bacterial cells, transfection for Eukaryotic cells, although insertion of a viral vector is often called **transduction**. Cloning vectors provide a backbone for the DNA insert to be reproduced and propagated in Bacteria, however, these vectors are only useful for storing a genetic sequence. By themselves, they are incapable of allowing for transcription and translation of the gene into a functional Protein product.

Characteristics of Cloning Vectors

A vector must possess the following characteristics:

1. The cloning vectors used often have elements necessary for their propagation and maintenance in *Escherichia coli*, such as a functional Origin of Replication (ORI). The ***Col*E1** origin of replication is found in many plasmids. All cloning vectors have features that allow a gene to be conveniently inserted into the vector or removed from it. This may be a Multiple Cloning Site (MCS) or polylinker, which contains many unique restriction sites. The restriction sites in the MCS are first cleaved by restriction enzymes, then a PCR-amplified target gene also digested with the same enzymes is ligated into the vectors using DNA ligase. The target DNA sequence can be inserted into the vector in a specific direction if so desired. The restriction sites may be further used for sub-cloning into another vector if necessary.
2. Other cloning vectors may use Topoisomerase, instead of ligase and cloning may be done more rapidly without the need for restriction digest of the vector or insert. In this 'Topo Cloning' method a linearized vector is activated by attaching Topoisomerase I to its ends, and this 'TOPO-Activated' vector may then accept a PCR product by ligating both the 5' ends of the PCR product, releasing the Topoisomerase and forming a circular vector in the process. Another method of cloning without the use of DNA digest and ligase is by DNA recombination, for example as used in the Gateway Cloning System. The Gene, once Cloned into the Cloning Vector (called Entry Clone in this method), may be conveniently introduced into a variety of expression vectors by recombination.
3. A selectable marker is carried by the vector to allow the selection of positively transformed cells. Antibiotic resistance is often used as marker, an example being the Beta-Lactamase Gene, which confers resistance to the Penicillin group of Beta-Lactam Antibiotics like Ampicillin. Some vectors contain two selectable markers, for example the Plasmid pACYC177 has both Ampicillin and Kanamycin Resistance Gene. Shuttle vector which is designed to be maintained in two different organisms may also require two selectable markers, although some selectable markers, such as resistance to Zeocin and Hygromycin B are effective in different cell types. Auxotrophic selection markers that allow an auxotrophic organism to grow in minimal growth medium may also be used, examples of these are LEU2 and URA3 which are used with their corresponding Auxotrophic Strains of Yeast.

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4. Reporter genes are used in some cloning vectors to facilitate the screening of successful clones by using features of these genes that allow successful clone to be easily identified. Such features present in cloning vectors may be the ***IacZa*** fragment for **a** complementation in blue-white selection, and/or marker gene or reporter genes in frame with and flanking the MCS to facilitate the production of Fusion Proteins. Examples of fusion partners that may be used for screening are the Green Fluorescent Protein (GFP) and Luciferase.
5. A cloning vector need not contain suitable elements for the expression of a cloned target gene, such as a promoter and Ribosomal Binding Site (RBS), many however do, and may then work as an expression vector. The target DNA may be inserted into a site that is under the control of a particular promoter necessary for the expression of the target gene in the chosen host. Where the promoter is present, the expression of the gene is preferably tightly controlled and inducible so that Proteins are only produced when required.
6. Some vectors are designed for transcription only with no Heterologous Protein expressed, for example for in-vitro mRNA production. These vectors are called Transcription Vectors. They may lack the sequences necessary for polyadenylation and termination, therefore may not be used for Protein production.

Figure 2.1 illustrates a typical cloning vector, pBR322 one of the first plasmid widely used as cloning vector.

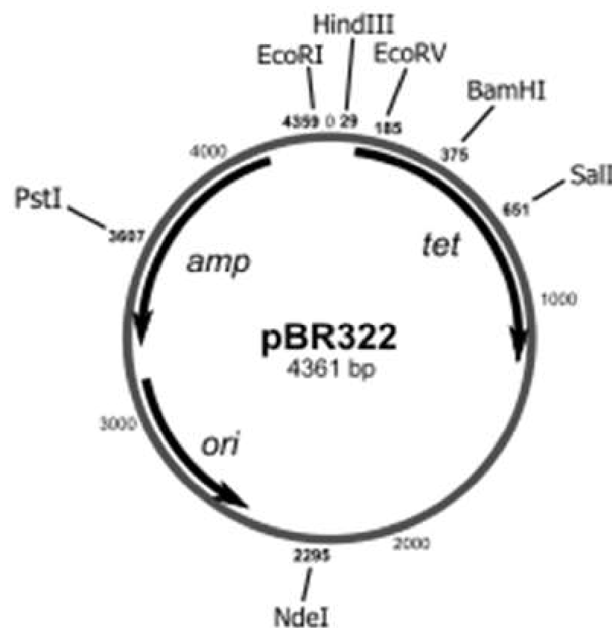


Fig. 2.1 A Typical Cloning Vector, pBR322 - One of the First Plasmid Widely used as Cloning Vector

Types of Cloning Vector

A large number of cloning vectors are available, and choosing the vector may depends a number of factors, such as the size of the insert, copy number and cloning method. Large insert may not be stably maintained in a general cloning vector, especially for those with a high copy number, therefore cloning large fragments may require more specialized cloning vector. The examples of Cloning Vectors are Plasmids, Bacteriophage, P1 Vectors, Phagemids, Cosmids, Bacterial Artificial Chromosome (BAC), Yeast Artificial Chromosome (YAC), Human Artificial Chromosome (HAC), and Retroviral Vectors.

1. Plasmids

Plasmids are double-stranded extra chromosomal and generally circular DNA sequences that are capable of replication using the host cell's replication machinery. Plasmid vectors minimalistically consist of an origin of replication that allows for semi-independent replication of the plasmid in the host. Plasmids are found widely in many bacteria, for example in *Escherichia coli*, but may also be found in a few Eukaryotes, for example in Yeast, such as *Saccharomyces cerevisiae*. Bacterial plasmids may be conjugative/ transmissible and non-conjugative. The conjugative plasmids mediate DNA transfer through conjugation and therefore spread rapidly among the bacterial cells of a population, for example F Plasmid, many R and some Col Plasmids. Whereas non-conjugative plasmids do not mediates DNA through conjugation for example, many R and Col Plasmids. Figure 2.2 illustrates in-situ genomic and plasmids DNA in bacteria.

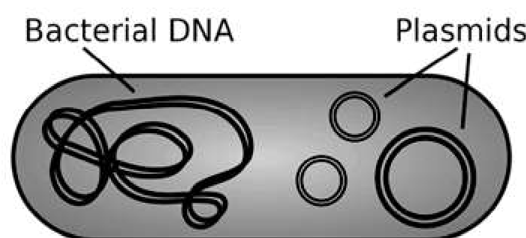


Fig. 2.2 *In-Situ Genomic and Plasmids DNA in Bacteria*

Plasmids with specially-constructed features are commonly used in laboratory for cloning purposes. These plasmids are generally non-conjugative but may have many more features, notably a 'Multiple Cloning Site (MCS)' where multiple restriction enzyme cleavage sites allow for the insertion of a transgene insert. The bacteria containing the plasmids can generate Plasmids are autonomously replicating circular extra-chromosomal DNA. They are the standard cloning vectors and the ones most commonly used. Most general plasmids may be used to clone DNA insert of up to 15 kb in size. One of the earliest commonly used cloning vector is the pBR322 plasmid. Other cloning vectors include the **pUC** series of

Plasmids, and a large number of different Cloning Plasmid Vectors are available. Many plasmids have high copy number, for example pUC19 which has a copy number of 500-700 copies per cell, and high copy number is useful as it produces greater yield of recombinant plasmid for subsequent manipulation. However low copy number plasmids may be preferably used in certain circumstances, for example, when the Protein from the cloned gene is toxic to the cells. Figure 2.3 illustrates the pUC Plasmids that contain a Multiple Cloning Site (Polylinker) and Gene for Amp Screening.

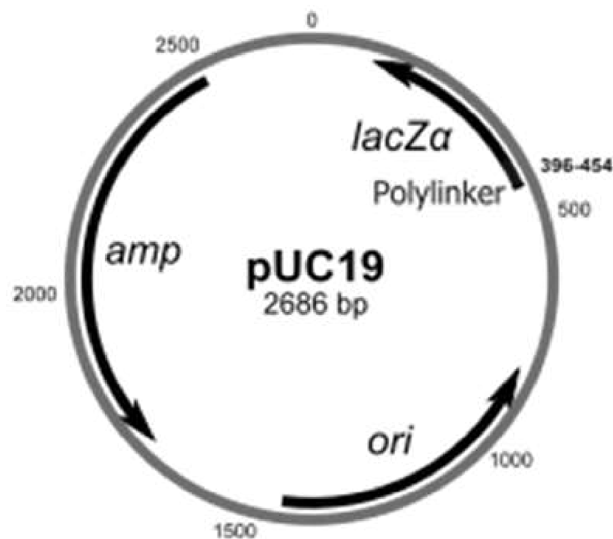


Fig. 2.3 *pUC Plasmids Contain a Multiple Cloning Site (Polylinker) and Gene for Amp Screening*

Characteristics of Plasmids

Plasmids were the first vectors to be used in gene cloning. They are naturally occurring and autonomously replicating extra-chromosomal double-stranded circular DNA molecules. However, not all plasmids are circular in origin. They are present in Bacteria, Archaea, and Eukaryotes. The size of plasmids ranges from 1.0 kb to 250 kb. DNA insert of up to 10 kb can be cloned in the plasmids. The plasmids have high copy number which is useful for production of greater yield of recombinant plasmid for subsequent experiments. The low copy number plasmids are exploited under certain conditions like the cloned gene produces the Protein which is toxic to the cells. Plasmids only encode those Proteins which are essential for their own replication. These Protein-encoding genes are located near the ORI. Examples include pBR322, pUC18, F Plasmid, Col Plasmid. The pBR322 cloning vector has the following elements:

p = Plasmid

B = Bolivar (Name of the Scientist)

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R = Rodriguez (Name of the Scientist)

322 = Number of Plasmid Discovered in the Same Lab.

Figure 2.4 illustrates the physical map of pACYC184 vector.

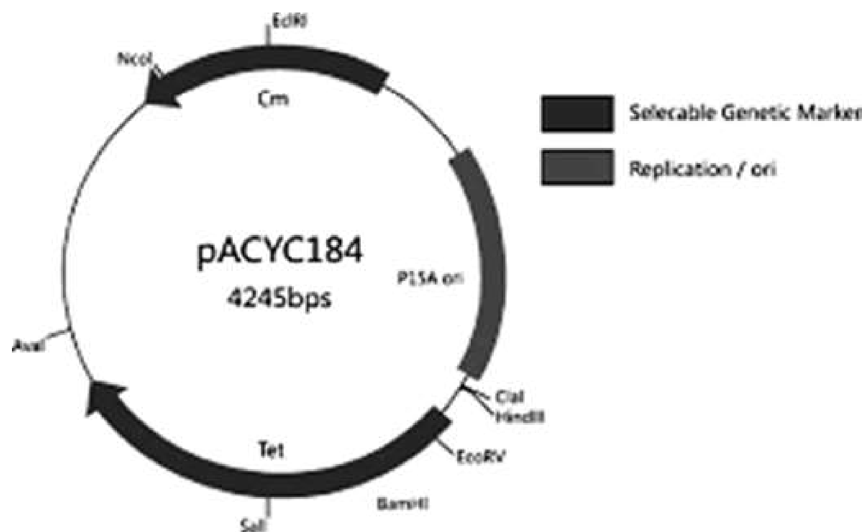


Fig. 2.4 Physical Map of pACYC184 Vector

Advantages of Plasmids as Vectors

1. Easy to manipulate and isolate because of small size.
2. More stable because of circular configuration.
3. Replicate independent of the host.
4. High copy number.
5. Detection easy because of antibiotic-resistant genes.

Disadvantages of Plasmids as Vectors

1. Large fragments cannot be cloned.
2. Size range is only 0 to 10 kb.
3. Standard methods of transformation are inefficient.

2. Bacteriophage

A bacteriophage also known informally as a **Phage** is a **Virus** that infects and replicates within Bacteria and Archaea. Bacteriophages are composed of Proteins that encapsulated a DNA or RNA genome, and may have relatively simple or elaborate structures. Their genomes may encode as few as 4 genes and as many as hundreds of genes. Phages replicate within the bacterium following the injection of their genome into its cytoplasm. Bacteriophages are among the most common and diverse entities in the biosphere. Bacteriophages are ubiquitous Viruses, found wherever bacteria exist. It is estimated there are more than

10^{31} bacteriophages on the planet, more than every other organism on Earth, including bacteria, combined. Phages are widely distributed in locations populated by bacterial hosts, such as Soil or the Intestines of Animals. One of the densest natural sources for phages and other Viruses is Seawater, where up to 9 x 10^8 virions per ml have been found in microbial mats at the surface, and up to 70% of Marine Bacteria may be infected by phages. They have been used for over 90 years as an alternative to antibiotics in the former US and Central Europe as well as in France. They are seen as a possible therapy against multi-drug resistant strains of many bacteria. Figure 2.5 illustrates the structure of Bacteriophage Lambda (Phage l).

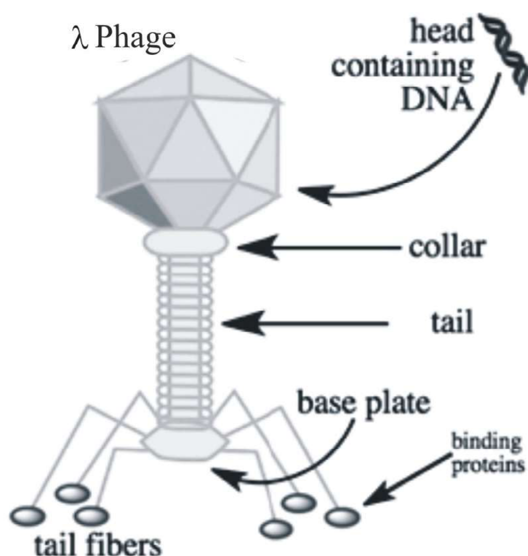


Fig. 2.5 *Structure of Bacteriophage Lambda (Phage l)*

The bacteriophages used for cloning are the **phage** λ and **M13 phage**. There is an upper limit on the amount of DNA that can be packed into a phage (a maximum of 53 kb), therefore to allow foreign DNA to be inserted into phage DNA, phage cloning vectors may need to have some non-essential genes deleted, for example the genes for lysogeny since using phage λ as a cloning vector involves only the lytic cycle. There are two kinds of λ phage vectors - Insertion Vector and Replacement Vector. Insertion vectors contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted. In replacement vectors, the cleavage sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted. There is also a lower size limit for DNA that can be packed into a phage, and vector DNA that is too small cannot be properly packaged into the phage. This property can be used for selections, vector without insert may be too small therefore only vectors with insert

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may be selected for propagation. Figure 2.6 illustrates the physical map of lambda bacteriophage (phage λ).

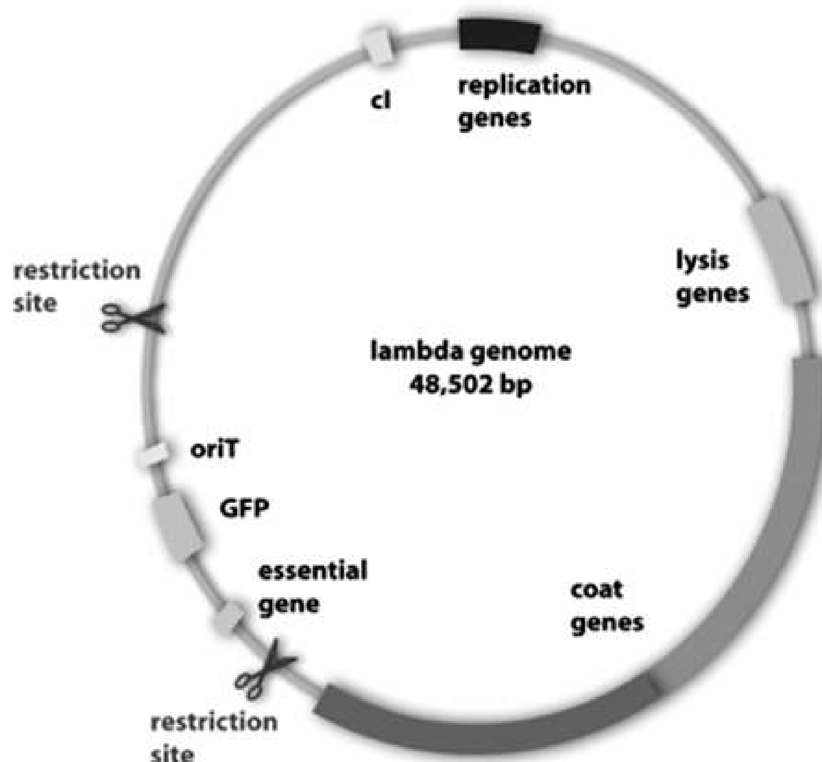


Fig. 2.6 Physical Map of Lambda Bacteriophage (Phage λ)

Thus phage Lambda (Phage λ) is a Phage (Virus) Genome with about 48502 bp size. The 1/3rd of the Bacteriophage Genome is non-essential, so that it can be cut, removed and replaced by donor DNA fragment during cloning. It can recombinant only 4-5 kbp of donor DNA fragment. The **Phage λ** has head, tail, and tail fibers. Its genome consists of 48.5 kb of DNA and 12 bp ssDNA which comprise of sticky ends at both the terminals. Since these ends are complementary, they are cohesive and also referred to as Cos Sites. Infection by λ phage requires adsorption of tail fibers on the cell surface, contraction of the tail, and injection of the DNA inside the cell. Figure 2.7 illustrates the replication of phage λ in *Escherichia coli*.

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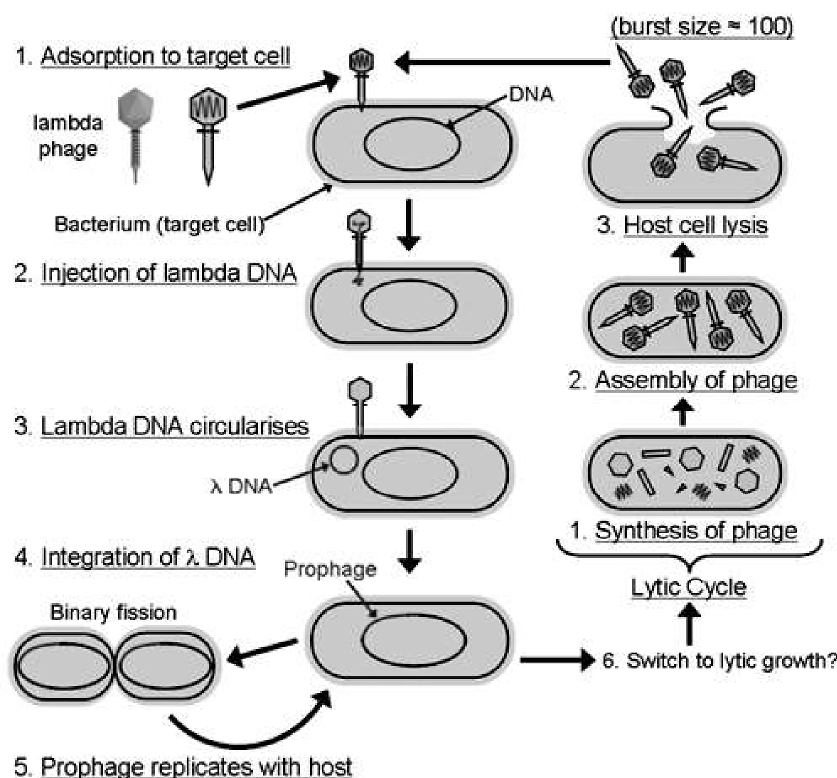


Fig. 2.7 Replication of Phage λ in Escherichia Coli

Whereas, **the M13 Phage vectors** are used for obtaining single-stranded copies of the cloned DNA. They are utilized in DNA sequencing and in-vitro mutagenesis. The M13 phages are derived from Filamentous Bacteriophage M13. The genome of M13 is 6.4 kb. DNA inserts of large sizes can be cloned. From the double-stranded inserts, pure single-stranded DNA copies are obtained.

Figure 2.8 illustrates the multiplication of Phage M13 in *Escherichia coli*.

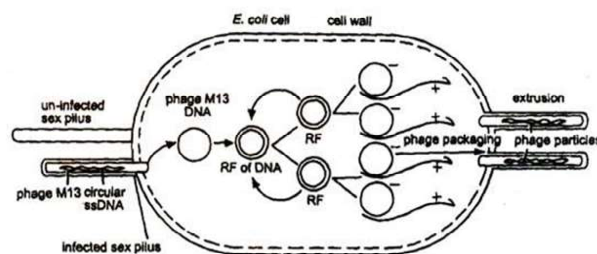


Fig. 2.8 *Multiplication of Phage M13 in Escherichia Coli*

P1 Phage Vector: This cloning vector system is based on the Bacteriophage P1 (Sternberg, 1990). The capabilities of the P1 cloning system are intermediate between those of Cosmids and YACs. The size of fragments cloned into P1 vectors averages from 75 to 95 kb, but fragments as large as 100 kb can be cloned. The efficiency of cloning for P1 is about 10^5 clones per microgram of

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vector and insert DNA. The P1 cloning vector includes two P1 DNA origins of replication: a plasmid origin of replication that maintains plasmid DNA in *Escherichia coli* at 1 copy per host chromosome and a lytic origin of replication that, when induced, amplifies the copy number of the P1 vector to about 20 copies per cell. In addition to selectable markers, the vector contains the ***pac*** site and two ***loxP*** sites of phage P1. Recombinant molecules are packaged in-vitro using various P1 packaging extracts. Packaging begins with a cut made at the ***pac*** (packaging) site. DNA is packaged by a headful mechanism, with 100–115 kb of DNA fitting into a P1 phage head. The first cut for packaging is made at the ***pac*** site, the second cut is not sequence specific but is at a fixed distance from the ***pac*** site. The linear DNA is injected into the recipient bacterium. Only those DNA molecules that contain two ***loxP*** sites are able to circularize and replicate in the bacterium. The ***loxP*** sites are specific DNA sequences from phage P1 upon which a P1 site-specific recombinase **Cre** acts. Figure 2.9 illustrates the physical map of P1 Cloning Vector.

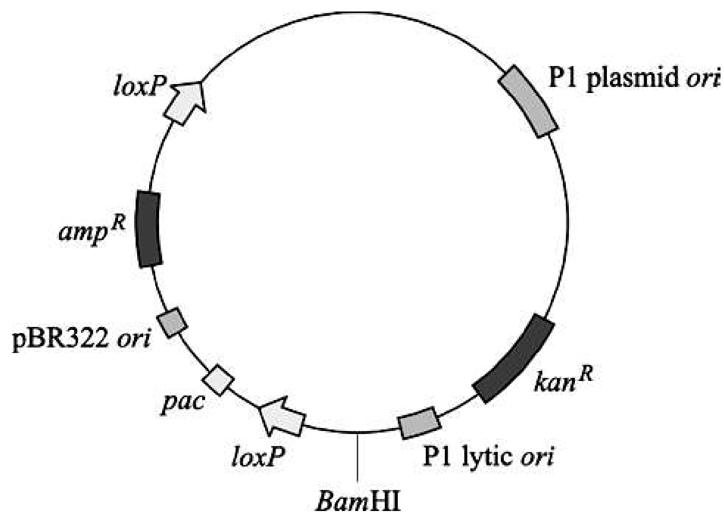


Fig. 2.9 *Physical Map of P1 Cloning Vector*

Types of Phage Vectors

There are following two types of phage vectors:

1. **Insertion Vectors:** These contain a particular cleavage site where the foreign DNA of up to 5-11 kb can be inserted. Figure 2.10 illustrates the cloning of an Insertion Vector.

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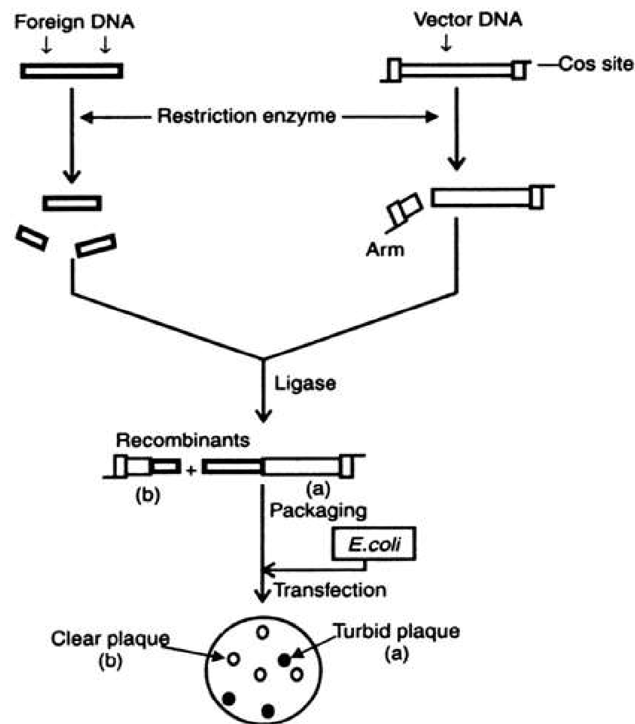


Fig. 2.10 Cloning of an Insertion Vector

2. **Replacement Vectors:** The cleavage sites flank a region which contains genes not necessarily important for the host, and these genes can be deleted and replaced by the DNA insert. Figure 2.11 illustrates the cloning of a Replacement Vector.

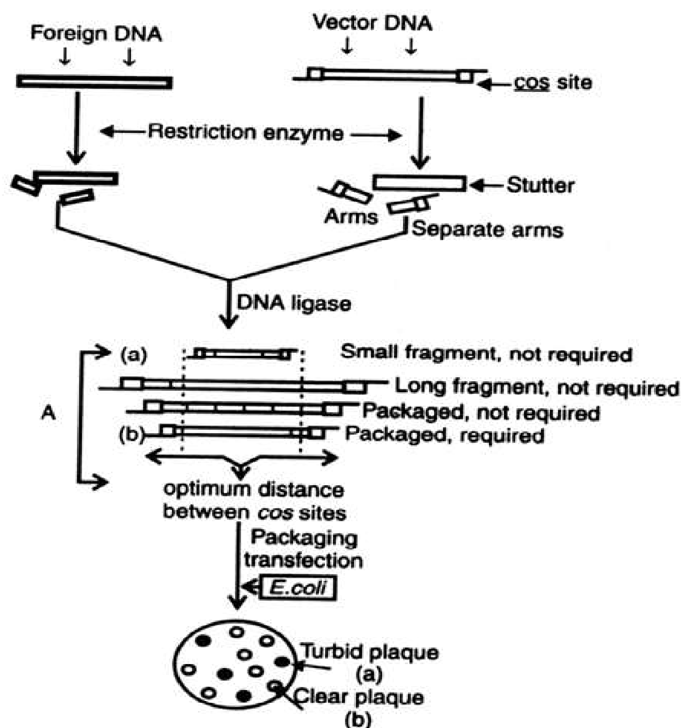


Fig. 2.11 *Cloning of a Replacement Vector*

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Advantages of using Phage Vectors

1. They are more efficient than Plasmids for Cloning large inserts.
2. Screening of phage plaques is much easier than identification of Recombinant Bacterial Colonies.

3. Phagemids or Phasmid Vectors

Sometimes generation of a single stranded DNA becomes important for DNA sequencing and site-directed mutagenesis. Using a single stranded DNA containing bacteriophage M13, a series of cloning vectors were developed. The bacteriophage **Fl** is closely related to M13 which also infects *Escherichia coli*. Phagemids are plasmids that contain Origin of Replication (ORI) for single strand DNA containing bacteriophage, such as F1. *Escherichia coli* maintains a plasmid as double stranded DNA due to plasmid as double stranded DNA due to plasmid ORI gene. If *Escherichia coli* cells are infected by the helper F1 phage, the ORI of F1 is activated. It switches to a mode of replication and produces single stranded DNA which is packed into phage particles as they are extruded from the host cell. The Figure 2.12 shows a phagemid pBlueScript II KS (+–) which is used for generation of single stranded DNA molecules. Thus phagemids are prepared artificially, contains the F1 origin of replication from F1 phage, generally used as a cloning vector in combination with M13 phage, and replicates as a plasmid and gets packaged in the form of single-stranded DNA in viral particles.

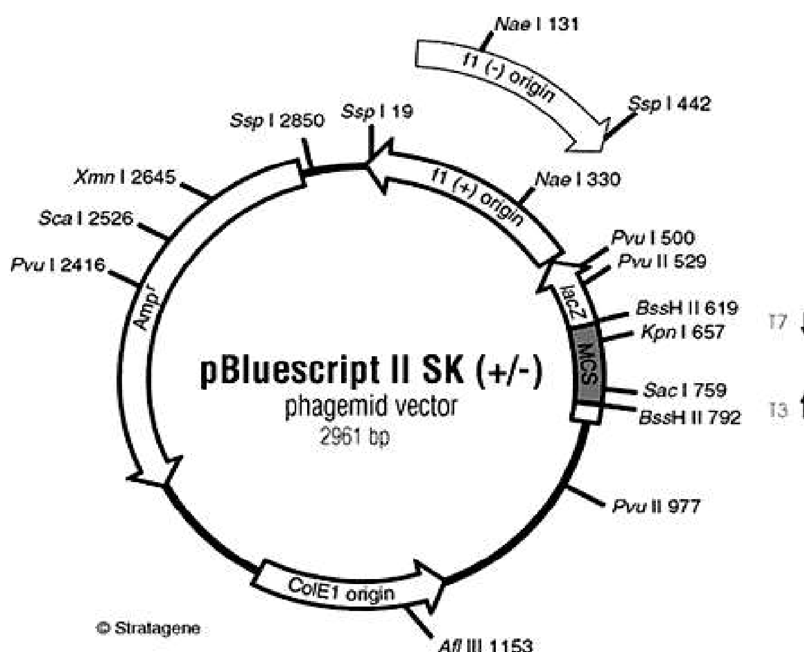


Fig. 2.12 Physical Map of a Phagemid pBlueScript II KS (+–)

Advantages of using Phagemids

1. They contain multiple cloning sites. 2. An inducible Lac Gene promoter is present. 3. Blue-White colony selection is observed.

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4. Cosmid Vectors

Cosmids are the specially designed plasmid vectors which have Cos sites. One such example of Cosmid is pJB8. This Cosmid is developed from a plasmid by addition of the lambda DNA with the Cos site. Cos sites are the particular sequence which is identified by the Phage endonuclease and cleave during the rolling circle replication. The size of the vector is 5.4 kb. For the in-vitro packaging (or even normal phage DNA assembly into the Protein coat), the size of the DNA needs to be in the range of 37 to 52 kb. Less than or more than the range size of the DNA would not allow the packaging of the DNA into the Protein coat and result in the formation of empty heads. Thus cosmids are plasmids, capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (Cos sites) which are necessary for efficient packaging of DNA into λ phage particles. They are also packaged into λ. This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction. Figure 2.13 illustrates the physical map of a Cosmid, pJB8 (5.4 kb).

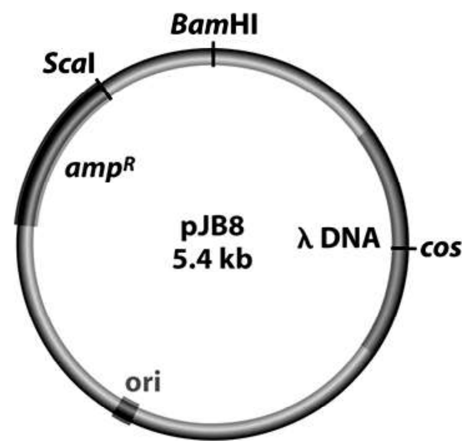


Fig. 2.13 *Physical Map of a Cosmid, pJB8 (5.4 kb)*

The Cosmids are plasmids that incorporate a segment of bacteriophage λ DNA that has the Cohesive End Site (Cos) which contains elements required for packaging DNA into λ particles. It is normally used to clone large DNA fragments between 28 and 45 kb. A Cosmid is a type of hybrid plasmid that contains a lambda phage Cos sequence.

Cosmids (Cos Sites + Plasmid = Cosmids)

DNA sequences are originally from the lambda phage. Cosmids can be used to build genomic libraries. They were first described by Collins and Hohn in 1978. Cosmids can contain 37 to 52 (normally 45) kb of DNA, limits based on the normal bacteriophage packaging size. They can replicate as plasmids if they have a suitable ORI, for example SN40 ORI in mammalian cells, ***col*E1** ORI for double-stranded DNA replication, or F1 ORI for single-stranded DNA replication in

Prokaryotes. They frequently also contain a gene for selection, such as antibiotic resistance, so that the transformed cells can be identified by plating on a medium containing the antibiotic. Those cells which did not take up the cosmid would be unable to grow. Unlike plasmids, they can also be packaged in phage capsids, which allow the foreign genes to be transferred into or between cells by transduction. Plasmids become unstable after a certain amount of DNA has been inserted into them, because their increased size is more conducive to recombination. To circumvent this, phage transduction is used instead. This is made possible by the cohesive ends, also known as Cos sites. In this way, they are similar to using the lambda phage as a vector, except 'All' the lambda genes have been deleted with the exception of the Cos sequence. The Cos sequences are about 200 bp long and essential for packaging. They contain a CosN site where DNA is nicked at each strand, 12 bp apart, by terminase. This causes linearization of the circular cosmid with two 'Cohesive' or 'Sticky Ends' of 12 bp. The DNA must be linear to fit into a phage head. The CosB site holds the terminase while it is nicking and separating the strands. The CosQ site of next cosmid is held by the terminase after the previous cosmid has been packaged, to prevent degradation by cellular DNases.

Figure 2.14 illustrates the cloning of a cosmid vector in *Escherichia coli*.

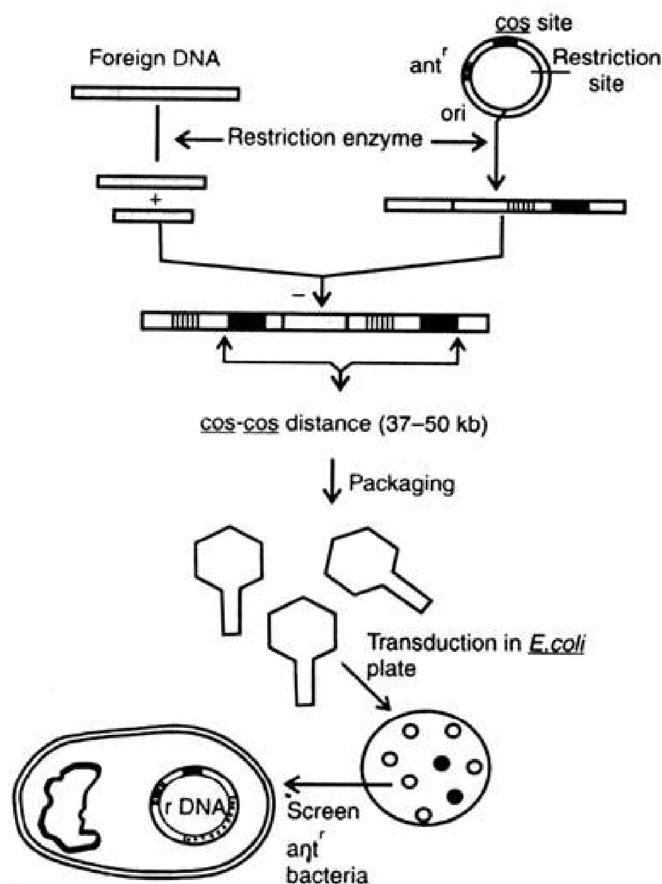


Fig. 2.14 *Cloning of a Cosmid Vector in Escherichia Coli*

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Advantages of using Cosmids as Vectors

1. They have high transformation efficiency and are capable of producing a large number of clones from a small quantity of DNA.
2. Also, they can carry up to 45 kb of insert compared to 25 kb carried by plasmids and the λ.

Disadvantages of using Cosmids as Vectors

Cosmids cannot accept more than 50 kb of the insert.

Figure 2.15 illustrates the physical map of SuperCosI Cosmid Vector (7.6 kb).

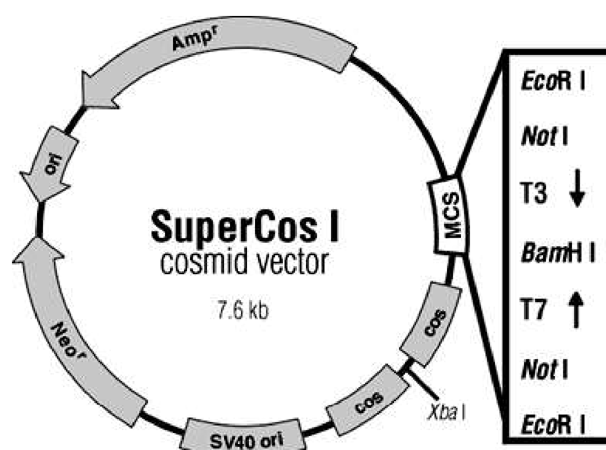


Fig. 2.15 Physical Map of SuperCosI Cosmid Vector (7.6 kb)

5. Bacterial Artificial Chromosomes (BACs)

Bacterial Artificial Chromosomes (BACs) are similar to *Escherichia coli* plasmid vectors. They contain ORI and genes which encode ORI binding Proteins. These Proteins are critical for BAC replication. It is derived from naturally occurring F plasmid. The DNA insert size varies between 150 to 350 kb. It means insert size of up to 350 kb can be cloned in Bacterial Artificial Chromosome (BAC). BACs are maintained in *Escherichia coli* with a copy number of only 1 per cell. BACs are based on F plasmid, another artificial chromosome called the PAC is based on the P1 phage. One example of the BAC is '**pUvBBAC**'. It is artificially synthesized plasmid with 11827 bp size. It is modification of bacterial F plasmid with cloning limit 35-300 kb. The chloramphenicol resistant gene and lactose metabolizing gene, the ***lacZ***, are the marker genes.

Figure 2.16 illustrates the physical map of Bacterial Artificial Chromosome (BAC) cloning vector 'pUvBBAC'.

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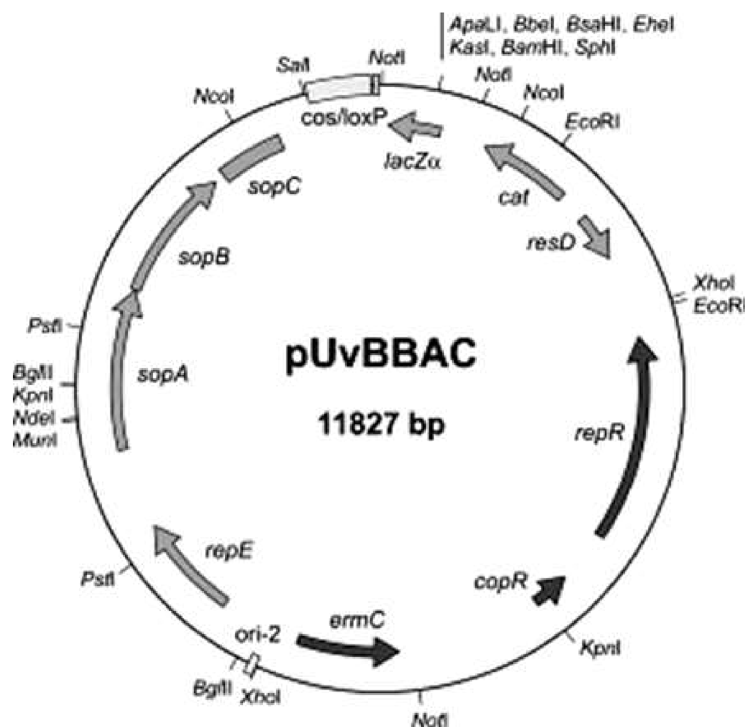


Fig. 2.16 The Physical Map of Bacterial Artificial Chromosome (BAC) Cloning Vector 'pUvBBAC'

Advantages of BACs

1. They are capable of accommodating large sequences without any risk of rearrangement.
2. BACs are frequently used for studies of genetic or infectious disorders.
3. High yield of DNA clones is obtained.

Disadvantages of BACs

1. They are present in low copy number.
2. The Eukaryotic DNA inserts with repetitive sequences are structurally unstable in BACs often resulting in deletion or rearrangement.

6. Yeast Artificial Chromosomes (YACs)

A large DNA insert of up to 200 kb can be cloned and carried by Yeast Artificial Chromosome (YAC). They are used for cloning inside Eukaryotic cells. These act as Eukaryotic chromosomes inside the host Eukaryotic cell. It possesses the Yeast telomere at each end. A Yeast Centromere Sequence (CEN) is present which allows proper segregation during Meiosis. The ORI is Bacterial in origin. Both Yeast and Bacterial cells can be used as Hosts.

Advantages of using YACs

1. A large amount of DNA can be cloned.
2. Physical maps of large genomes like the human genome can be constructed.

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Disadvantages of using YACs

1. Overall transformation efficiency is low.
2. The yield of cloned DNA is also low.

Figure 2.17 illustrates the physical map of Yeast Artificial Chromosome (YAC) cloning vector pYAC3.

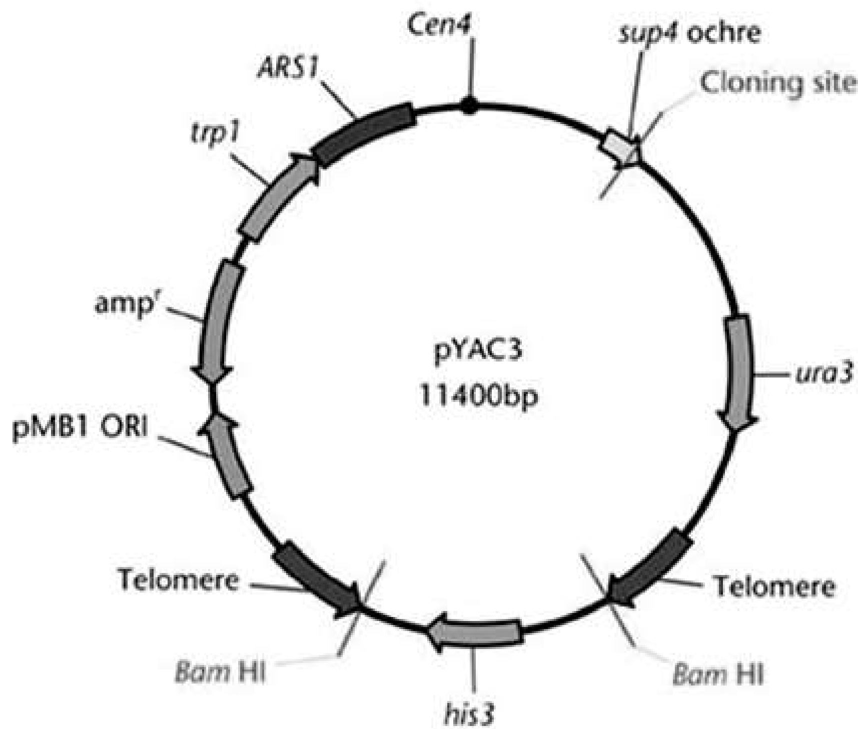


Fig. 2.17 *Physical Map of Yeast Artificial Chromosome (YAC) Cloning Vector, pYAC3*

Advantages of BACs over YACs

1. Comparatively stable.
2. Easy to transform.
3. Simple purification required.
4. User friendly.
5. Aid in the development of vaccines.

7. Human Artificial Chromosome (HAC)

Human Artificial Chromosome (HAC) may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function. It can carry very large DNA fragment (there is no upper limit on size for practical purposes), therefore it does not have the problem of limited cloning capacity of other vectors, and it also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral

vector. HAC is artificially synthesized chromosome used to transfer human gene. HAC constructed by minimum DNA elements for the maintenance of chromosome function. It enables gene introduction of desired sequences. There is no cloning limit, and it can carry large segments of DNA. They are used extensively in expression studies and determining the function of the human chromosomes.

Figure 2.18 illustrates the Human Artificial Chromosome (HAC) Cloning Vector.

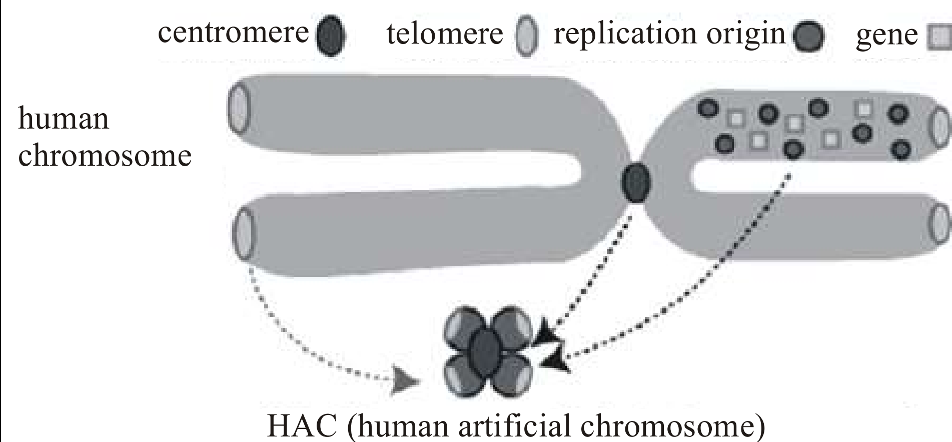


Fig. 2.18 *Human Artificial Chromosome (HAC) Cloning Vector*

Advantages of using HACs

1. No upper limit on DNA that can be cloned.
2. It avoids the possibility of insertional mutagenesis.

8. Retroviral Vectors

The **viral vectors** are generally genetically engineered Viruses carrying modified viral DNA or RNA that has been rendered noninfectious, but still contain viral promoters and also the transgene, thus allowing for translation of the transgene through a viral promoter. However, because viral vectors frequently are lacking infectious sequences, they require helper Viruses or packaging lines for large-scale transfection. Viral vectors are often designed for permanent incorporation of the insert into the host genome, and thus leave distinct genetic markers in the host genome after incorporating the transgene. For example, **retroviruses** leave a characteristic retroviral integration pattern after insertion that is detectable and indicates that the viral vector has incorporated into the host genome. Figure 2.19 illustrates the physical map of Gene Of Interest (GOI) in Retroviral Vector.

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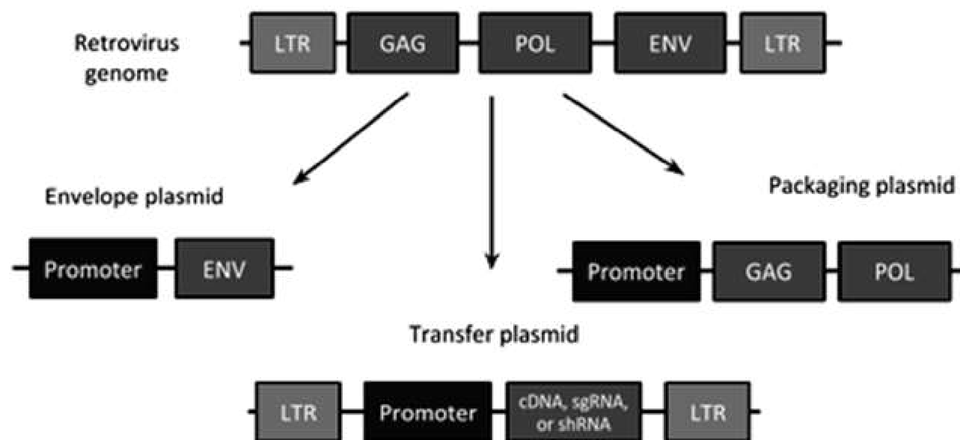


Fig. 2.19 Physical Map of Gene Of Interest (GOI) in Retroviral Vector

The retroviruses are the Virus with RNA as the genetic material. Retroviral vectors are used for introduction of novel or manipulated genes into the animal or human cells. The viral RNA is converted into DNA with the help of reverse transcriptase and henceforth, efficiently integrated into the host cell. Any gene of interest can be introduced into the retroviral genome. This Gene of Interest (GOI) can then integrate into host cell chromosome and reside there. They are widely used as a tool to study and analyze oncogenes and other human genes. Figure 2.20 illustrates the Nucleic Acid delivery and multiplication of Retrovirus.

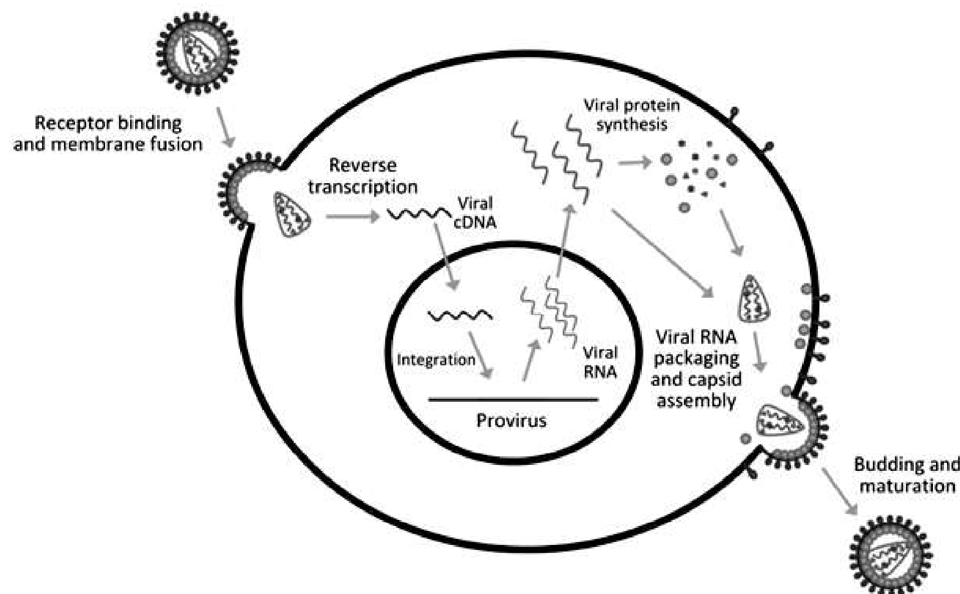


Fig. 2.20 *Nucleic Acid Delivery and Multiplication of Retrovirus*

9. Expression Vector

Expression vectors produce Proteins through the transcription of the vector's insert followed by translation of the mRNA produced, they therefore require more components than the simpler transcription only vectors. Expression in different host organism would require different elements, although they share similar requirements, for example a promoter for initiation of transcription, a ribosomal binding site for translation initiation, and termination signals. Example includes the Eukaryotic Expression Vector pSG5 with size 4100 bp. Thus the Eukaryotic vector is modified in such a way that it can be expressed in Prokaryotic cell known as Expression Vector, and allows RNA polymerase to transcribe genes.

Prokaryotes Expression Vector: It require sequences that encode for:

1. **Promoter:** Commonly used inducible promoters are promoters derived from ***lac*** operon and the T7 promoter. Other strong promoters used include ***trp*** promoter and ***lac*** promoter which are a hybrid of both the ***trp*** and ***lac*** Operon promoters.
2. **Ribosome Binding Site (RBS):** It follows the promoter, and promotes efficient translation of the Protein of interest.
3. **Translation Initiation Site:** It is Shine-Dalgarno sequence enclosed in the RBS, 8 base pairs upstream of the AUG start codon.

Eukaryotes Expression Vector: It require sequences that encode for:

1. **Polyadenylation Tail:** It creates a polyadenylation tail at the end of the transcribed pre-mRNA that protects the mRNA from exonucleases and ensures transcriptional and translational termination that stabilizes mRNA production.
2. **Minimal UTR Length:** UTRs contain specific characteristics that may impede transcription or translation, and thus the shortest UTRs or none at all are encoded for in optimal expression vectors.
3. **Kozak Sequence:** Vectors should encode for a Kozak sequence in the mRNA, which assembles the ribosome for translation of the mRNA.

Figure 2.21 illustrates the physical map of Eukaryotic Expression Vector, pSG5.

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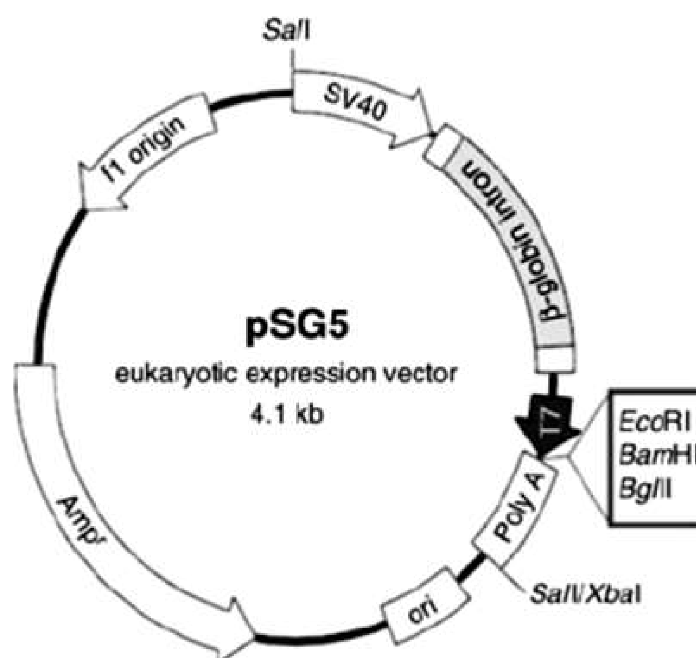


Fig. 2.21 *Physical Map of Eukaryotic Expression Vector, pSG5.*

10. Shuttle Vector

A **shuttle vector** is a vector (usually a plasmid) constructed so that it can propagate in two different host species. Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types. The main advantage of these vectors is they can be manipulated in *Escherichia coli*, then used in a system which is more difficult or slower to use, for example Yeast. The shuttle vectors include plasmids that can propagate in Eukaryotes and Prokaryotes, for example *Saccharomyces cerevisiae* and *Escherichia coli* or in different species of bacteria, such as both *Escherichia coli* and *Rhodococcus erythropolis*. There are also **adenovirus shuttle vectors**, which can propagate in *Escherichia coli* and mammals.

Shuttle vectors are frequently used to quickly make multiple copies of the gene in *Escherichia coli* (amplification). They can also be used for in-vitro experiments and modifications, for example mutagenesis, PCR. One of the most common types of shuttle vectors is the **yeast shuttle vector**. Almost all commonly used *Saccharomyces cerevisiae* vectors are shuttle vectors. Yeast shuttle vectors have components that allow for replication and selection in both *Escherichia coli* cells and Yeast cells. The *Escherichia coli* component of a Yeast shuttle vector includes an origin of replication and a selectable marker, for example Antibiotic Resistance, Beta Lactamase, Beta Galactosidase. The Yeast component of a Yeast shuttle vector includes an Automatically Replicating Sequence (ARS), a Yeast Centromere (CEN), and a Yeast selectable marker, for example URA3, a gene that encodes an enzyme for Uracil Synthesis (Lodish *et al.* 2007). Figure 2.22 illustrates the physical map of Shuttle Vector for Yeasts.

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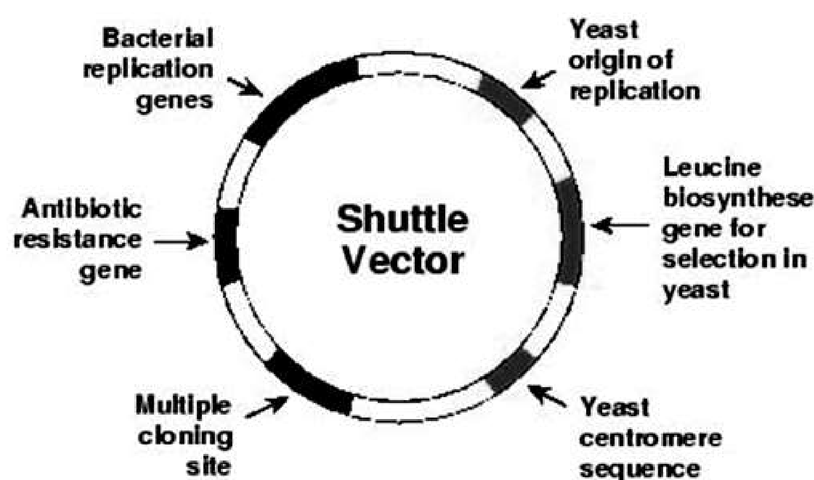


Fig. 2.22 Physical Map of Shuttle Vector for Yeasts

Thus shuttle vectors can survive in two different organisms, and include two origins of replication, one for each organism, and two genes for selection, one for each organism. Researchers often wish to move vectors from one organism to another. The shuttle vector is useful for performing such movements but must have a few features that allow it to survive within different organisms. The shuttle vector must contain an origin of replication for both organisms, as these are sequences that are recognized differently by Proteins from different species. If the shuttle vector is to be used in a Eukaryote, such as Yeast, then it must also contain a centromere sequence so that microtubules can bind to it and it is properly segregated during cell division.

Finally, two selective markers must be used, one for each organism. Antibiotics are usually specific to bacterial cells, but have no effect on Eukaryotic cells such as Yeast. If Yeast is a desired organism for the shuttle vector, then some other selectable marker must be used. Often, strains defective in certain metabolic pathways are used. If the Yeast strain is defective in one enzyme for the manufacture of an Amino Acid, then the cells are not able to grow without being given exogenous Amino Acid. However, if the functional copy of the DNA for the defective enzyme is present on the shuttle vector, then the Yeast can now grow without the addition of the specific Amino Acid. The metabolic differences give researchers a phenotypic difference between cells that have the vector and those that do not have it.

Several well-known shuttle vectors have been successfully used to transform obligatory anaerobic thermophiles. All these vectors feature antibiotic resistance genes originating from mesophilic Gram-Positive Bacteria. Vector pIKM1, based on pUC19, contains the ***mls*** gene from pIM13 and the thermostable kanamycin-resistance cassette from plasmid pKD102 found in *Streptococcus faecalis* (a ruminant mesophile capable of growth at 45°C). Suicide vectors for gene knockout were developed based on pIKM1.

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Figure 2.23 illustrates the physical map of pIKM1 shuttle vector.

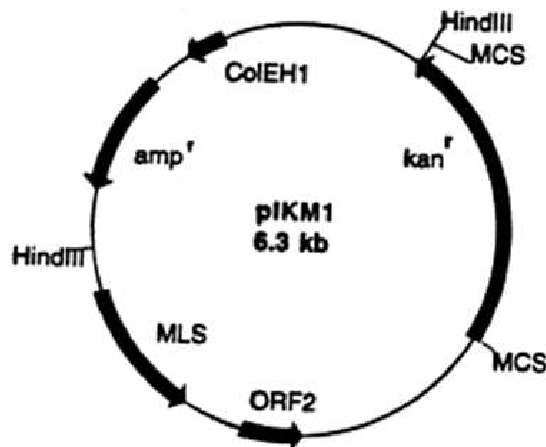


Fig. 2.23 *Physical Map of pIKM1 Shuttle Vector*

11. Ti and Ri Plasmids

Agrobacterium species harboring Tumor inducing (Ti) or hairy Root inducing (Ri) plasmids cause crown gall or hairy root diseases, respectively, in plants. *Agrobacterium tumefaciens* is a plant pathogen that induces tumor on a wide variety of dicotyledonous plants and the disease is caused by Tumor inducing plasmid (pTi). Similarly *Agrobacterium rhizogenes* is a plant pathogen that induces hairy roots on a wide variety of dicotyledonous plants and the disease is caused by Root inducing plasmid (pRi). Virulence ***(Vir)*** genes of Ri as well as of Ti plasmids are essential for the T DNA transfer into plant chromosomes. These natural plasmids provide the basis for vectors to make transgenic plants. The plasmids are approximately 200 kbp in size. Both pTi and pRi are unique in following two respects:

1. They contain some genes, located within their T DNA, which have regulatory sequences recognized by plant cells, while their remaining genes have Prokaryotic regulatory sequences.
2. Both plasmids naturally transfer a part of their DNA, the T DNA, into the host genome, which makes *Agrobacterium* a natural genetic engineer.

The complete sequence analysis confirms that the pathogenic plasmids contain gene clusters for DNA replication, virulence, T DNA, opine utilization and conjugation. T DNA genes have lower G + C content, which is presumably suitable for expression in host plant cells. Besides these genes, each plasmid has a large number of unique genes. Even plasmids of the same opine type differ considerably in gene content and are highly chimeric in structures. The plasmids seem to interact with each other and with plasmids of other members of the *Rhizobiaceae* and are likely to shuffle genes of infection between Ti and Ri plasmids. Plasmid stability genes are talked about, which are important for plasmid evolution and construction of useful strains.

Ti Plasmid: The Ti plasmid refers to a type of plasmid that allows *Agrobacterium tumefaciens* to infect plants, producing a Tumor/Crown Gall Tumor. Thus, the Ti plasmid consists of both Tumor Forming and Virulence (*Vir*) Genes. The *Vir* genes are responsible for the transfer of T DNA that contains the tumor-forming genes into the plant genome by excision and integration. A typical Ti plasmid consists of four regions: Region A (T DNA responsible for Tumor Formation), Region B (responsible for Replication), Region C (responsible for Conjugation), and Region D (responsible for Virulence). The genes in the T DNA region controls the production of plant growth hormones (Auxin and Agropine), inducing the proliferation of the infected plant cells. Both ends of the T DNA region are flanked by a border sequence that contains direct sequence repeats of 24 base pairs. Only the right border is indispensable for T DNA transfer.

Thus a Ti or tumour inducing plasmid is a plasmid that often, but not always, is a part of the genetic equipment that *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* use to transduce their genetic material to plants. The Ti plasmid is lost when *Agrobacterium* is grown above 28°C. Such cured bacteria do not induce crown galls, i.e., they become a virulent. The pTi and pRi share little sequence homology but are functionally rather similar. The Ti plasmids are classified into different types based on the type of opine produced by their genes. The different opines specified by pTi are Octophine, Nopaline, Succiamopine and Leucinopine. The plasmid has 196 Genes that code for 195 Proteins. There is a structural RNA. The plasmid is 206,479 nucleotides long, the GC content is 56% and 81% of the material is coding genes. There are no pseudogenes. The modification of this plasmid is very important in the creation of transgenic plants. The Ti plasmid contains all the genes which required for tumor formation. Virulence genes (*Vir* genes) are also located on the Ti plasmid. The *Vir* genes encode a set of Proteins responsible for the excision, transfer and integration of the T DNA into the plant nuclear genome. The basic elements of the vectors designed for *Agrobacterium* mediated transformation that were taken from the native Ti-plasmid.

- The **T DNA border sequences**, at least the right border, which initiates the integration of the T DNA region into the plant genome.
- The *Vir* **genes,** which are required for transfer of the T DNA region to the plant. The *Vir*A codes for a receptor which reacts to the presence of phenolic compounds, such as Acetosyringone, Syringealdehyde or Acetovanillone which leak out of damaged plant tissues. The *Vir*B encodes Proteins which produce a pore/pilus-like structure. The *Vir*C binds the overdrive sequence. The *Vir*D1 and *Vir*D2 produce endonucleases which target the direct repeat borders of the T DNA segment, *Vir*D4 is the coupling Protein. The *Vir*E binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes, beginning with the right border. The *Vir*G activates *Vir* gene expression after binding to a consensus sequence, once it has been phosphorylated by *Vir*A.

- A **modified T DNA region** of the Ti plasmid, in which the genes responsible for tumor formation are removed by genetic engineering and replaced by foreign genes of diverse origin, such as from plants, bacteria, Virus. When these genes are removed, transformed plant tissues or cells regenerate into normal-appearing plants and, in most cases, fertile plants.

Figure 2.24 illustrates the physical map of Ti plasmid.

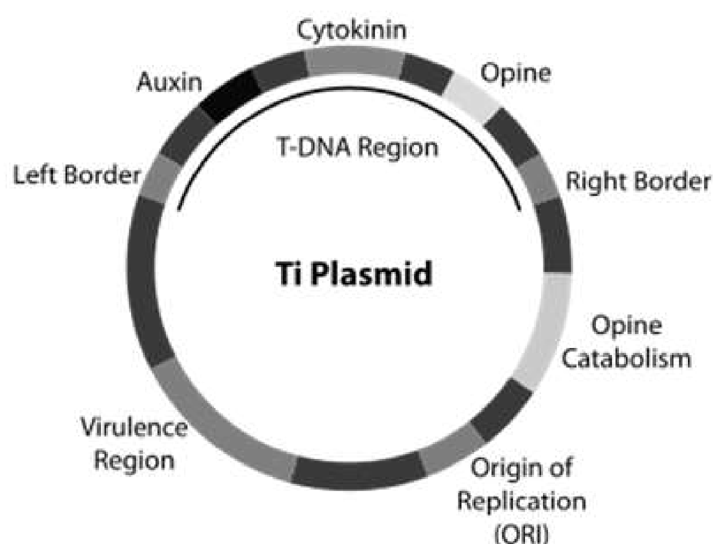


Fig. 2.24 *Physical Map of Ti Plasmid*

Ri Plasmid: The Ri plasmid refers to a type of plasmid that allows *Agrobacterium rhizogenes* to infect pant cells by producing hairy roots. It consists of two T DNA fragments known as Tr DNA and T1 DNA. Both fragments are separated by a 15 kb DNA segment. The Tr DNA is similar to the T DNA region of the Ti plasmid and contains genes that induce the production of Growth Hormones (GH). The T1 DNA region consists of four genes: ***rolA*** (responsible for the formation of hairy roots), ***rolB*** (induces root initiation and callus formation), ***rolC*** (promotes root growth), and ***rolD*** (suppresses callus growth). The transformation of T DNA is induced by the ***Vir*** genes. Similarly to the Ti plasmid, a defined region of DNA of the Ri plasmid, the transferred or T DNA, is transferred from the bacteria to the infected plant cell and integrated into the plant genome. The **Ri plasmids have been less studied than the Ti plasmids**. Like the Ti plasmid, Ri plasmids can be characterized by their opine specificity, Agropine or Mannopine.

(i) There are similarities in sequence between Ti and Ri plasmids, most notably in the T DNA and the virulence (*Vir*) regions.

(ii) Transfer of the T DNA from Ri plasmids to the plant cell most probably uses the same mechanism as T DNA transfer from Ti plasmids.

(iii) Vectors for transferring foreign DNA to plant cells based on Ri plasmids have been developed for use in plant genetic engineering.

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Figure 2.25 illustrates the formation of hairy roots by *Agrobacterium rhizogenes.*

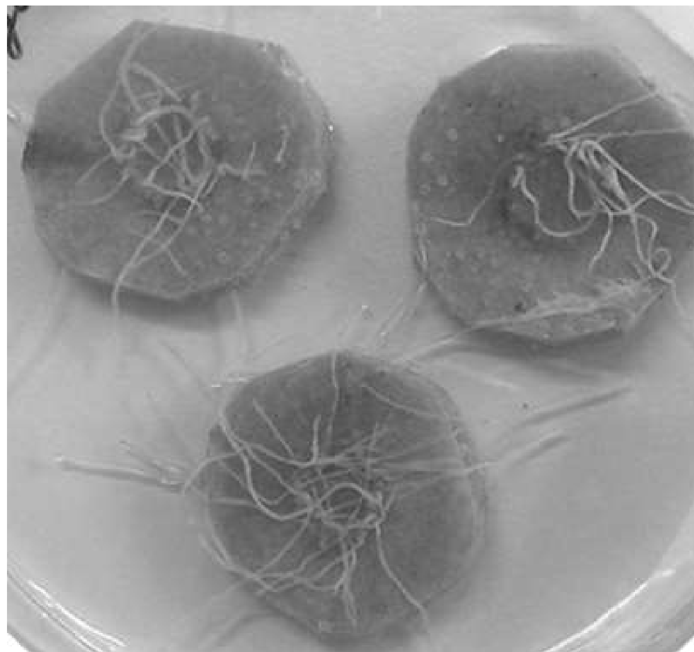


Fig. 2.25 *Formation of Hairy Roots by Agrobacterium Rhizogenes*

Similarities between Ti and Ri Plasmids

- Ti and Ri plasmids are naturally produced by *Agrobacterium.*
- Both types of plasmids cause diseases in many dicotyledonous plants.
- Both Ti and Ri plasmids transfer a part of the plasmid DNA known as T DNA into the plant genome with the aid of virulence (*Vir*) genes.
- The size of both Ti and Ri plasmids are around 200 kb.
- The regulatory sequences of some genes found in the T DNA of both plasmids are recognized by the plant cellular mechanisms.
- The other genes in the T DNA consist of Prokaryotic regulatory sequences.
- Due to the ability to infect plant cells, both Ti and Ri plasmids are used in genetic engineering to produce transgenic plants.

Differences between Ti and Ri Plasmid

Ti plasmid is a type of plasmid of that allows the bacteria to infect plants, producing a tumor/crown gall tumor. Whereas Ri plasmid is a type of plasmid that allows bacteria to infect plant cells by producing hairy roots. *Agrobacterium* species are plant pathogens that induce various diseases in higher plants. Ti (Tumor inducing) and Ri (Root inducing) plasmids are two types of natural plasmids produced by *Agrobacterium* sp. Ti plasmids are produced by *Agrobacterium tumefaciens* while Ri plasmids are produced by *Agrobacterium rhizogenes.* Both

Ti and Ri plasmids consist of a part of the plasmid DNA known as T DNA that is transferred into the plant genome with the aid of virulence (*Vir*) genes. The main difference between Ti and Ri plasmid is that Ti plasmid induce tumor/crown gall in dicots whereas Ri plasmid induces hairy roots. Due to their ability to infect plants, both Ti and Ri plasmids are widely used as vectors to produce transgenic plants.

Table 2.4 illustrates the comparative account of Ti plasmid vs. Ri plasmid.

Table 2.1 *Comparative Account of Ti Plasmid vs. Ri Plasmid*

S. No.	Ti Plasmid	Ri Plasmid
1	Ti plasmid is a type of plasmid of that allows the bacteria to infect plants, producing a tumor/crown gall tumor.	Ri plasmid is a type of plasmid of that allows the bacteria to infect plant cells by producing hairy roots.
2	Produced by *Agrobacterium tumifaciens.*	Produced by *Agrobacterium rhizogenes.*
3	Size is 180-205 kb.	Size is 250 kb.
4	Induces the formation of crown gall/tumor.	Induces the formation of hairy roots.
5	Less studied compared to Ri plasmids.	Well studied.

Check Your Progress

1. What do you understand by the cloning vector?
2. Elaborate on the plasmids.
3. Define the bacteriophage.
4. Explain the phagemids or phasmid vectors.
5. Illustrate the cosmid vectors.
6. State the bacterial artificial chromosomes (BACs).
7. Interpret the yeast artificial chromosomes (YACs).
8. Define the term human artificial chromosome (HAC).
9. What do you mean by the shuttle vector?
10. Differentiate between the Ti and Ri plasmid.

2.3 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. The cloning vector is a small piece of DNA, taken out of a virus, a bacteria or the cell of a higher organism, and can be stably maintained into a foreign DNA fragment for cloning purposes. The vector, therefore, contains characters that allow for convenient insertion or removal of a DNA fragment to or from vector.

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2. Plasmids are double-stranded extra chromosomal and generally circular DNA sequences that are capable of replication using the host cell's replication machinery. Plasmid vectors minimalistically consist of an origin of replication that allows for semi-independent replication of the plasmid in the host.
3. A bacteriophage also known informally as a phage is a virus that infects and replicates within bacteria and Archaea. Bacteriophages are composed of proteins that encapsulated a DNA or RNA genome, and may have relatively simple or elaborate structures. Their genomes may encode as few as 4 genes and as many as hundreds of genes.
4. Phagemids are plasmids that contain Origin of Replication (ORI) for single strand DNA containing bacteriophage, such as F1. Escherichia coli maintains a plasmid as double stranded DNA due to plasmid as double stranded DNA due to plasmid ORI gene.
5. Cosmids are the specially designed plasmid vectors which have Cos sites. One such example of Cosmid is pJB8. This Cosmid is developed from a plasmid by addition of the lambda DNA with the Cos site. Cos sites are the particular sequence which is identified by the Phage endonuclease and cleave during the rolling circle replication.
6. Bacterial Artificial Chromosomes (BACs) are similar to Escherichia coli plasmid vectors. They contain ORI and genes which encode ORI binding proteins. These proteins are critical for BAC replication. It is derived from naturally occurring F plasmid.
7. A large DNA insert of up to 200 kb can be cloned and carried by Yeast Artificial Chromosome (YAC). They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell. It possesses the yeast telomere at each end.
8. Human Artificial Chromosome (HAC) may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function.
9. A shuttle vector is a vector (usually a plasmid) constructed so that it can propagate in two different host species. Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types.
10. Ti plasmid is a type of plasmid of that allows the bacteria to infect plants, producing a tumour/ crown gall tumour. Whereas Ri plasmid is a type of plasmid that allows bacteria to infect plant cells by producing hairy roots. Agrobacterium species are plant pathogens that induce various diseases in higher plants. Ti (Tumour inducing) and Ri (Root inducing) plasmids are two types of natural plasmids produced by Agrobacterium sp. Ti plasmids are produced by Agrobacterium tumefaciens while Ri plasmids are produced by Agrobacterium Rhizogenes.

2.4 SUMMARY

NOTES

- The cloning vector is a small piece of DNA, taken out of a virus, a bacteria or the cell of a higher organism, and can be stably maintained into a foreign DNA fragment for cloning purposes.
- The vector therefore contains characters that allow for convenient insertion or removal of a DNA fragment to or from vector.
- Plasmids are double-stranded extra chromosomal and generally circular DNA sequences that are capable of replication using the host cell's replication machinery.
- Plasmid vectors minimalistically consist of an origin of replication that allows for semi-independent replication of the plasmid in the host.
- A bacteriophage also known informally as a phage is a virus that infects and replicates within bacteria and Archaea. Bacteriophages are composed of proteins that encapsulated a DNA or RNA genome, and may have relatively simple or elaborate structures. Their genomes may encode as few as 4 genes and as many as hundreds of genes.
- Phagemids are plasmids that contain Origin of Replication (ORI) for single strand DNA containing bacteriophage, such as F1. Escherichia coli maintains a plasmid as double stranded DNA due to plasmid as double stranded DNA due to plasmid ORI gene.
- Cosmids are the specially designed plasmid vectors which have Cos sites. One such example of Cosmid is pJB8. This Cosmid is developed from a plasmid by addition of the lambda DNA with the Cos site. Cos sites are the particular sequence which is identified by the Phage endonuclease and cleave during the rolling circle replication.
- Bacterial Artificial Chromosomes (BACs) are similar to Escherichia coli plasmid vectors. They contain ORI and genes which encode ORI binding proteins. These proteins are critical for BAC replication. It is derived from naturally occurring F plasmid.
- A large DNA insert of up to 200 kb can be cloned and carried by Yeast Artificial Chromosome (YAC). They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell.
- Human Artificial Chromosome (HAC) may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function.
- A shuttle vector is a vector (usually a plasmid) constructed so that it can propagate in two different host species. Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types.

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- Ti plasmid is a type of plasmid of that allows the bacteria to infect plants, producing a tumour/ crown gall tumour. Whereas Ri plasmid is a type of plasmid that allows bacteria to infect plant cells by producing hairy roots.
- Agrobacterium species are plant pathogens that induce various diseases in higher plants. Ti (Tumour inducing) and Ri (Root inducing) plasmids are two types of natural plasmids produced by Agrobacterium sp.
- Ti plasmids are produced by Agrobacterium tumefaciens while Ri plasmids are produced by Agrobacterium Rhizogenes.

2.5 KEY WORDS

- **Cloning vector:** The cloning vector is a small piece of DNA, taken out of a virus, a bacteria or the cell of a higher organism, and can be stably maintained into a foreign DNA fragment for cloning purposes.
- **Plasmids:** Plasmids are double-stranded extra chromosomal and generally circular DNA sequences that are capable of replication using the host cell's replication machinery.
- **Bacteriophage:** A bacteriophage also known informally as a phage is a virus that infects and replicates within bacteria and Archaea. Bacteriophages are composed of proteins that encapsulated a DNA or RNA genome, and may have relatively simple or elaborate structures.
- **Phagemids:** Phagemids are plasmids that contain Origin of Replication (ORI) for single strand DNA containing bacteriophage, such as F1. Escherichia coli maintains a plasmid as double stranded DNA due to plasmid as double stranded DNA due to plasmid ORI gene.
- **Cosmids:** Cosmids are the specially designed plasmid vectors which have Cos sites. One such example of Cosmid is pJB8. This Cosmid is developed from a plasmid by addition of the lambda DNA with the Cos site.
- **Bacterial artificial chromosomes:** Bacterial Artificial Chromosomes (BACs) are similar to Escherichia coli plasmid vectors. They contain ORI and genes which encode ORI binding proteins.
- **Human artificial chromosome:** Human Artificial Chromosome (HAC) may be potentially useful as a gene transfer vectors for gene delivery into human cells, and a tool for expression studies and determining human chromosome function.
- **Shuttle vector:** A shuttle vector is a vector (usually a plasmid) constructed so that it can propagate in two different host species. Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types.

2.6 SELF-ASSESSMENT QUESTIONS AND EXERCISES

NOTES

Short-Answer Questions

1. Elaborate on the cloning vector.
2. What do you understand by the plasmids?
3. Explain the term bacteriophage.
4. Define the phagemids or phasmid vectors.
5. Interpret the cosmid vectors.
6. State the bacterial artificial chromosomes (BACs).
7. Illustrate the yeast artificial chromosomes (YACs).
8. Explain the term human artificial chromosome (HAC).
9. Define the term shuttle vector.
10. What are the main differences between the Ti and Ri plasmid?

Long-Answer Questions

1. What are the cloning vectors? Explain different types of vectors.
2. Discuss the characteristics of plasmid, cosmid, and phagemids.
3. Analyse the yeast artificial chromosome with their properties.
4. Explain the bacterial artificial chromosome.
5. Describe the shuttle vectors. Give its mechanism.
6. Briefly define the yeast vectors with the help of examples.

2.7 FURTHER READINGS

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UNIT 3 GENE TRANSFER METHODS IN ANIMALS

NOTES

Structure

3.0 INTRODUCTION

Gene transfer technique is widely used in fundamental research and applied biology. The transfer of DNA into animal cells is a basic and customary procedure. Gene delivery experiments helps to precise the genetic construct (or transgene) in the recipient cells. There are several uses of gene transfer like large-scale commercial production of recombinant antibodies and vaccines and gene medicine or gene therapy, etc.

The gene transfer concept between the cells, was first revealed in bacteria. In 1928, first mechanism was discovered by Frederick Griffith, named as transformation in bacterium Streptococcus Pneumonia.

Gene transfer is the process of introducing foreign genetic material, such as DNA or RNA, into host cells. Gene transfer must reach the genome of the host cell to induce gene expression. Successful gene delivery requires the foreign gene delivery to remain stable within the host cell and can either integrate into the genome or replicate independently of it. This requires foreign DNA to be synthesized as part of a vector, which is designed to enter the desired host cell and deliver the transgene to that cell's genome. Vectors utilized as the method for gene delivery can be divided into two categories, recombinant viruses and synthetic vectors (viral and non-viral).

Gene transfer is a necessary step in gene therapy for the introduction or silencing of a gene to promote a therapeutic outcome in patients and also has applications in the genetic modification of crops. There are many different methods of gene transfer for various types of cells and tissues.

In this unit, you will study about the gene transfer methods in animals, electroporation, microinjection, biolistic particle delivery system, sonoporation, optical transfection, protoplast fusion, and retrovirus mediated gene transfer.

3.1 OBJECTIVES

After going through this unit, you will be able to:

- Define the gene transfer methods in animals
- Understand the electroporation, and microinjection,
- Elaborate on the biolistic particle delivery system
- Illustrate the sonoporation, optical transfection, and protoplast fusion
- Explain the retrovirus mediated gene transfer

3.2 GENE TRANSFER METHODS IN ANIMALS

Gene delivery refers to the process of introducing foreign genetic material such as DNA or RNA directly into host cells. Genetic material either DNA or RNA must reach the genome of the host cell in order to induce gene expression. However, this is not the end of process; successful gene delivery must ensure the foreign genetic material to stay stable within the host cell. Also, the foreign material either integrates into the genome or replicate independently of it.

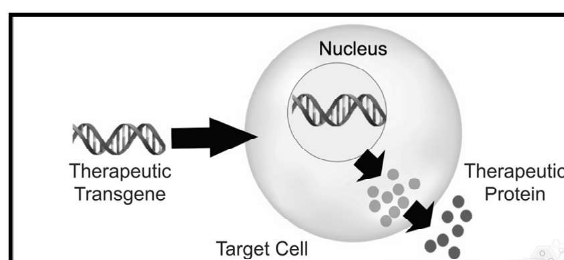


Fig. 3.1 *Principle of Gene Therapy*

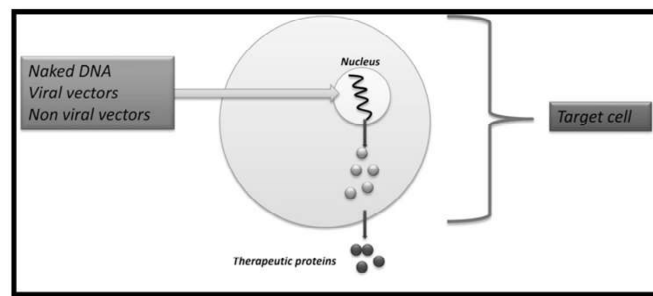


Fig. 3.2 *Mode of Gene Delivery*

Gene delivery is an essential step in gene therapy for introducing or silencing of a gene to encourage a therapeutic outcome in patients. It also serves in the genetic modification of crops. There are several methods of gene delivery system:

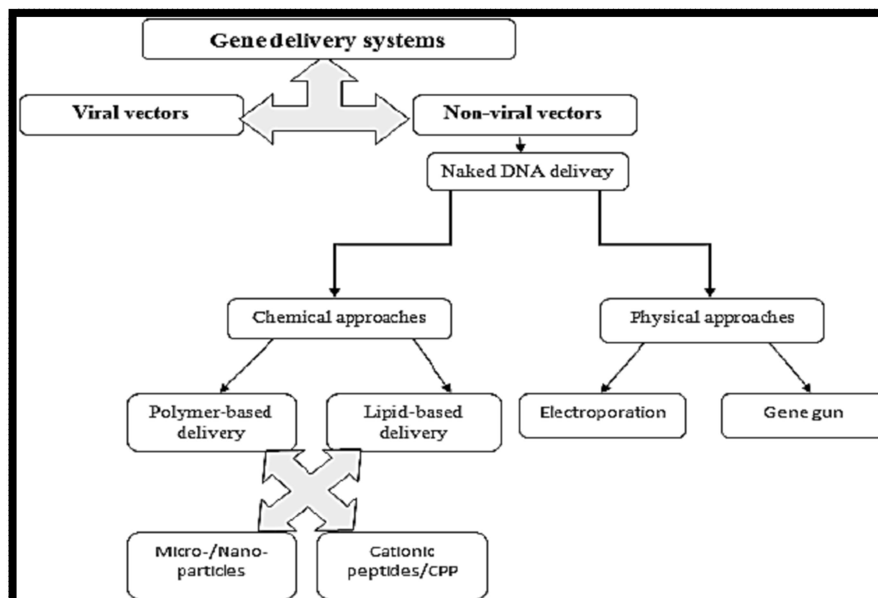


Fig. 3.3 *Different Gene Delivery Methods*

3.2.1 Electroporation

Electroporation is a method of promoting competence. Cells are briefly shocked with an electric field of 10-20 kV/cm, which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock, the holes are rapidly closed by the cell's membrane-repair mechanisms.

3.2.2 Microinjection

The use of microinjection as a biological procedure began in the early twentieth century, although even through the 1970s it was not commonly used. By the 1990s, its use had escalated significantly and it is now considered a common laboratory

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technique, along with vesicle fusion, electroporation, chemical transfection, and viral transduction, for introducing a small amount of a substance into a small target.

There are two basic types of microinjection systems. The first is called a constant flow system and the second is called a pulsed flow system. In a constant flow system, which is relatively simple and inexpensive though clumsy and outdated, a constant flow of a sample is delivered from a micropipette and the amount of the sample which is injected is determined by how long the needle remains in the cell. This system typically requires a regulated pressure source, a capillary holder, and either a coarse or a fine micromanipulator.

A pulsed flow system, however, allows for greater control and consistency over the amount of sample injected: the most common arrangement for intracytoplasmic sperm injection includes an Eppendorf "Femtojet" injector coupled with an Eppendorf "InjectMan", though procedures involving other targets usually take advantage of much less expensive equipment of similar capability. Because of its increased control over needle placement and movement and in addition to the increased precision over the volume of substance delivered, the pulsed flow technique usually results in less damage to the receiving cell than the constant flow technique. However, the Eppendorf line, at least, has a complex user interface and its particular system components are usually much more expensive than those necessary to create a constant flow system or than other pulsed flow injection systems.

3.2.3 Biolistic Particle Delivery System

In genetic engineering, biolistic particle delivery system or simply a gene gun is a tool used to deliver exogenous DNA (transgenes), RNA, or protein inside cells. In the biolistic approach, gene of interest or other biological molecule are coated on a heavy metal like gold or tungsten and then accelerated to high velocity via source such as helium pulse and finally driven through cell wall and membranes into the target. The biolistic or gene gun approach is easy to perform and can be applied on target such as tissues, bacteria, cell cultures, plants, animals, organs as well as organelles.

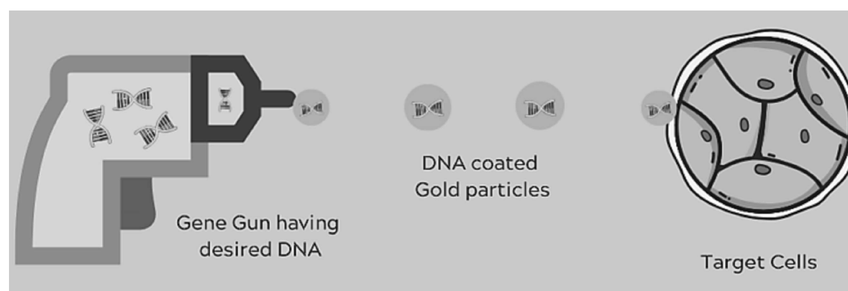


Fig. 3.4 *Biolistic Approach*

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Gene Gun Design

The gene gun which was originally an air pistol customized to fire dense tungsten particles. It was invented by John C Sanford, Ed Wolf, and Nelson Allen at Cornell University along with Ted Klein of DuPont between 1983 and 1986. Onions, due to their large size were chosen as the first target to deliver particles coated with a marker gene which would pass on a signal if appropriate insertion of the DNA transcript takes place.

The apparatus includes a chamber coupled to an outlet to generate vacuum. At the top, a cylinder (in which Helium gas flows) is temporarily sealed off from the rest of chamber with a plastic rupture disk. A microcarrier made of plastic is placed close to rupture disk. It consist of DNA coated tungsten particle, the microscopic pellets *(i.e.* coated microprojectiles). The target organs/organelles/ cells/tissue is placed in the apparatus. A stopping screen is positioned in between the target cells and microcarrier assembly.

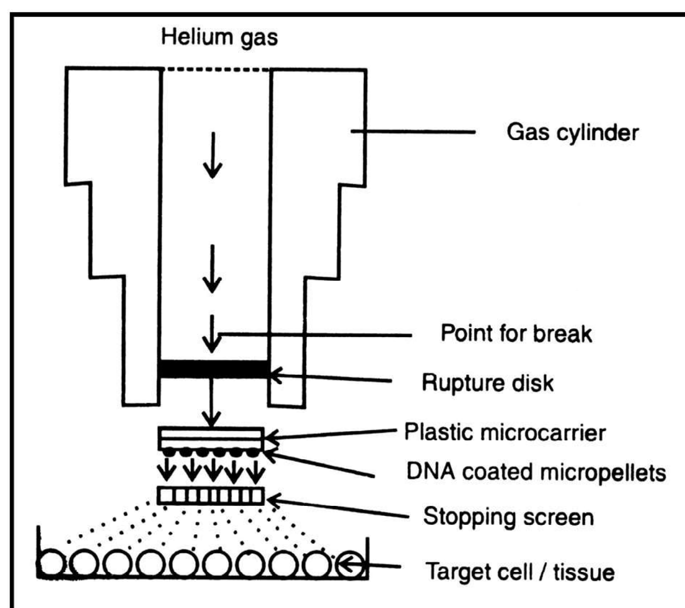


Fig. 3.5 *Working System of a Gene Gun*

Helium gas is then flown in to the cylinder at a very high velocity. Whenever the pressure of cylinder goes beyond the bursting point of plastic disk, it gets ruptured. Helium shock waves drive the plastic mircrocarrier containing the DNA coated micropellets. The stopping screen allows the micropellets to pass through and deliver DNA into target organs/organelles/cells/tissue. The transformed cells, i.e., cells having the foreign DNA are then regenerated onto nutrient rich medium. The regenerated plant tissues are selected over nutrient culture media containing cither herbicide or antibiotics. The selected plants are then evaluated or examined for expression of foreign DNA.

Advantages of Biolistic Approach

1. Gene gun delivery method is versatile, rapid and easy to use
2. In this method, transient or stable expression is possible
3. Very small amounts of nucleic acid either DNA or RNA or molecule of interest is required for efficient transformation
4. Very high levels of co-transformation are possible
5. Large DNA fragments may be transferred or small interfering RNAs (siRNAs) for gene silencing
6. Many cell types can be transfected, including non-dividing cells and plants
7. Potentially toxic treatments (such as from viruses or chemical- and lipid-based systems) are avoided
8. Plastid transformation has also seen great success with particle bombardment when compared to other current techniques, such as *Agrobacterium* mediated transformation

Applications of Biolistic Approach

- **Plant Systems:** Biolistic technology is particularly valuable for target cells or tissues that are noncompliant to other transformation methods like agriculturally significant monocotyledonous plants. Embryos, seedlings, leaves, cultured cells, floral tissues, epidermal tissues, apical meristems etc. are among several targets that have been successfully transformed.
- **Animal Systems:** Gun gene delivery system has been used effectively for the transformation of intact animal embryos, tissues, as well as animal cells in culture. Particle delivery is a suitable technique for transforming these sensitive cells as little pre- or post-bombardment manipulation is required.
- **Other Biological Systems:** Biolistic skills have also been applied to targets as diverse as mitochondria, bacteria, algae, chloroplasts, fungi and pollen. Gene gun has been further used to gain understanding of infectious disease, cancer and wound healing as well as to generate immune responses in animals. It is also used to assay gene expression and regulation both in vitro and in vivo.

Limitations of Biolistic Approach

1. Biolistic approach introduces target DNA or RNA or any molecule randomly into the target cells.
2. Thus, the foreign DNA, RNA or molecule of interest get transformed into whatever genomes are present in the cell, i.e., nuclear, plasmid or mitochondrial.

3.2.4 Sonoporation

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Sonoporation, or cellular sonication refers to the use of sound usually ultrasonic frequencies for altering the permeability of the target cell plasma membrane. This methodology is regularly used in molecular biology as well as non-viral gene therapy to enable the uptake of large molecules such as DNA or RNA into the target cell, in a cell disruption process referred to as transformation or transfection. Sonoporation technique utilizes the acoustic cavitation of micro bubbles to augment delivery of these large DNA or RNA molecules. However, it should be used skilfully as extensive exposure to low-frequency (<MHz) ultrasound has been reported to cause total cell rupturing (cellular death).

Sonoporator- Equipment used to carry out the process of sonoporation

High end molecular biology technique known as 'Sonoporation' is executed with a specific equipment known as sonoporator. 'Sonoporation' may also be executed with specially customized piezoelectric transducers which are connected to bench-top functional generators and acoustic amplifiers. For carrying out some specific application, standard ultrasound medical devices can also be used.

Micro Bubble Agents

Sonoporation uses micro bubbles for considerably enhancing the process of transfection and in some cases it is essential for DNA uptake. Micro Bubbles (MBs) are referred to as the bubbles smaller than one hundredth of a millimetre in diameter, however, larger than one micrometre. The composition of the bubble shell and filling material determine important design features such as buoyancy, crush strength, thermal conductivity, and acoustic properties.

Advantages of Sonoporation

- Sonoporation is under active study for the introduction of foreign genes in tissue culture cells, especially mammalian cells.
- It aids in delivery of plasmids to cells and tissues for gene therapy based research as well as studies.
- It is also used widely for delivery of nucleic acids such as RNAi, SiRNA to target cell/tissue/organs for studies on control of gene therapy as well as gene expression.
- It also aids in delivering chemotherapeutic agents to non-permeable target cells/tissue/organs.
- It aids in delivery of agents which helps in determining metabolic effects on cells/tissue/organs/organelles.

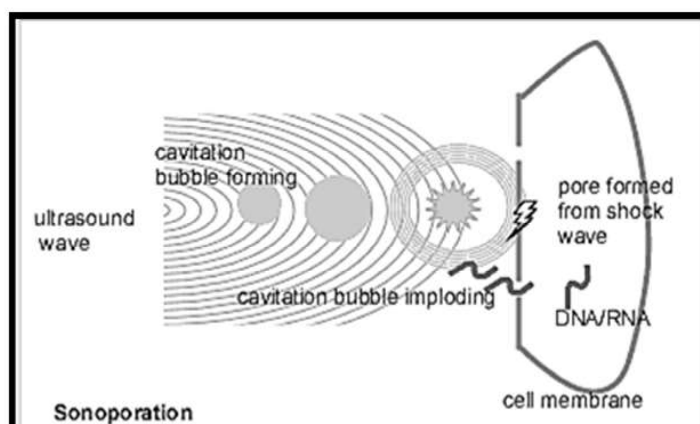


Fig. 3.6 *Basic Principle of Sonoporation*

3.3 OPTICAL TRANSFECTION

Optical transfection is the process of introducing nucleic acids into cells using light. Typically, a laser is focussed to a diffraction limited spot (~1 μm diameter) using a high numerical aperture microscope objective. The plasma membrane of a cell is then exposed to this highly focussed light for a small amount of time (typically tens of milliseconds to seconds), generating a transient pore on the membrane. The generation of a photopore allows exogenous plasmid DNA, RNA, organic fluorophores, or larger objects such as semiconductor quantum nanodots to enter the cell. In this technique, one cell at a time is treated, making it particularly useful for single cell analysis.

To put the above simply, cells do not usually allow certain types of substances into their interior space. Lasers can be used to burn a tiny hole on the cell surface, allowing those substances to enter. This is tremendously useful to biologists who are studying disease, as a common experimental requirement is to put things (such as DNA) into cells.

This technique was first demonstrated in 1984 by Tsukakoshi et al., who used a frequency tripled Nd: YAG to generate stable and transient transfection of normal rat kidney cells. Since this time, the optical transfection of a host of mammalian cell types has been demonstrated using a variety of laser sources, including the 405 *nm* continuous wave (cw), 488 *nm* cw, or pulsed sources such as the 800 *nm* femtosecond pulsed Ti:Sapphire or 1064 *nm* nanosecond pulsed Nd:YAG.

A typical optical transfection protocol is as follows:

1. Build an optical tweezers system with a high NA objective.
2. Culture cells to 50-60% confluency.

3. Expose cells to at least 10 μ*g*/*ml* of plasmid DNA.
4. Dose the plasma membrane of each cell with 10-40 *ms* of focussed laser, at a power of <100 *mW* at focus.
5. Observe transient transfection 24-96 *h* later.
6. Add selective medium if the generation of stable colonies is desired.

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3.4 PROTOPLAST FUSION

Protoplast fusion also called Somatic fusion, is a type of genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both, a somatic hybrid. Hybrids have been produced either between different varieties of the same species (e.g. between non-flowering potato plants and flowering potato plants) or between two different species (e.g. between wheat Triticum and rye Secale to produce Triticale).

Uses of somatic fusion include making potato plants resistant to potato leaf roll disease. Through somatic fusion, the crop potato plant Solanum Tuberosum – the yield of which is severely reduced by a viral disease transmitted on by the aphid vector – is fused with the wild, non-tuber-bearing potato Solanum Brevidens, which is resistant to the disease. The resulting hybrid has the chromosomes of both plants and is thus similar to polyploid plants. Somatic hybridization was first introduced by Carlson et al. in Nicotiana Glauca.

3.5 RETROVIRUS MEDIATED GENE TRANSFER

A retrovirus is a type of virus that inserts a copy of its RNA genome[a] into the DNA of a host cell that it invades, thus changing the genome of that cell. Once inside the host cell's cytoplasm, the virus uses its own reverse transcriptase enzyme to produce DNA from its RNA genome, the reverse of the usual pattern, thus retro (backwards). The new DNA is then incorporated into the host cell genome by an integrase enzyme, at which point the retroviral DNA is referred to as a provirus. The host cell then treats the viral DNA as part of its own genome, transcribing and translating the viral genes along with the cell's own genes, producing the proteins required to assemble new copies of the virus.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

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Gamma retroviral and lentiviral vectors for gene therapy have been developed that mediate stable genetic modification of treated cells by chromosomal integration of the transferred vector genomes. This technology is of use, not only for research purposes, but also for clinical gene therapy aiming at the long-term correction of genetic defects, e.g., in stem and progenitor cells. Retroviral vector particles with tropism for various target cells have been designed. Gamma retroviral and lentiviral vectors have so far been used in more than 300 clinical trials, addressing treatment options for various diseases. Retroviral mutations can be developed to make transgenic mouse models to study various cancers and their metastatic models.

Check Your Progress

1. Define gene delivery.
2. Differentiate between biolistic approach and sonoporation.
3. What are the limitations of biolistic approach?
4. Explain the micro bubbles.
5. What is a sonoporator?

3.6 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Gene delivery refers to the process of introducing foreign genetic material such as DNA or RNA directly into host cells. Genetic material either DNA or RNA must reach the genome of the host cell in order to induce gene expression. However, this is not the end of process; successful gene delivery must ensure the foreign genetic material to stay stable within the host cell. Also, the foreign material either integrates into the genome or replicate independently of it.
2. In genetic engineering, biolistic particle delivery system or simply a gene gun is a tool used to deliver exogenous DNA (transgenes), RNA, or protein inside cells. Sonoporation, or cellular sonication refers to the use of sound usually ultrasonic frequencies for altering the permeability of the target cell plasma membrane. This methodology is regularly used in molecular biology as well as non-viral gene therapy to enable the uptake of large molecules such as DNA or RNA into the target cell, in a cell disruption process referred to as transformation or transfection. Sonoporation technique utilizes the acoustic cavitation of micro bubbles to augment delivery of these large DNA or RNA molecules.

3. Biolistic approach has its own limitation like: - introduces target DNA or RNA or any molecule randomly into the target cells. Thus, the foreign DNA, RNA or molecule of interest get transformed into whatever genomes are present in the cell, i.e., nuclear, plasmid or mitochondrial.
4. Micro Bubbles (MBs) are referred to as the bubbles smaller than one hundredth of a millimetre in diameter, however larger than one micrometre. The composition of the micro bubble shell and filling material determine important design features such as buoyancy, crush strength, thermal conductivity, and acoustic properties.
5. High end molecular biology technique known as 'Sonoporation' is executed with a specific equipment known as sonoporator.

NOTES

3.7 SUMMARY

- Gene transfer technique is widely used in fundamental research and applied biology. The transfer of DNA into animal cells is a basic and customary procedure. Gene delivery experiments helps to precise the genetic construct (or transgene) in the recipient cells.
- The gene transfer concept between the cells, was first revealed in bacteria. In 1928, first mechanism was discovered by Frederick Griffith, named as transformation in bacterium Streptococcus Pneumonia.
- Gene delivery refers to the process of introducing foreign genetic material, such as DNA or RNA directly into host cells. Genetic material either DNA or RNA must reach the genome of the host cell in order to induce gene expression.
- Electroporation is a method of promoting competence. Cells are briefly shocked with an electric field of 10-20 kV/cm, which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock, the holes are rapidly closed by the cell's membrane-repair mechanisms.
- In genetic engineering, biolistic particle delivery system or simply a gene gun is a tool used to deliver exogenous DNA (transgenes), RNA, or protein inside cells.
- Sonoporation, or cellular sonication refers to the use of sound usually ultrasonic frequencies for altering the permeability of the target cell plasma membrane. This methodology is regularly used in molecular biology as well as non-viral gene therapy to enable the uptake of large molecules such as DNA or RNA into the target cell, in a cell disruption process referred to as transformation or transfection.

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- Optical transfection is the process of introducing nucleic acids into cells using light. Typically, a laser is focussed to a diffraction limited spot (~1 μm diameter) using a high numerical aperture microscope objective.
- Protoplast fusion also called Somatic fusion, is a type of genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both, a somatic hybrid.
- A retrovirus is a type of virus that inserts a copy of its RNA genome[a] into the DNA of a host cell that it invades, thus changing the genome of that cell.

3.8 KEY WORDS

- **Gene delivery:** Gene delivery refers to the process of introducing foreign genetic material such as DNA or RNA directly into host cells.
- **Electroporation:** Electroporation is a method of promoting competence. Cells are briefly shocked with an electric field of 10-20 kV/cm, which is thought to create holes in the cell membrane through which the plasmid DNA may enter.
- **Sonoporation:** Sonoporation, or cellular sonication refers to the use of sound usually ultrasonic frequencies for altering the permeability of the target cell plasma membrane.
- **Optical transfection:** Optical transfection is the process of introducing nucleic acids into cells using light. Typically, a laser is focussed to a diffraction limited spot (~1 μm diameter) using a high numerical aperture microscope objective.
- **Protoplast fusion:** Protoplast fusion also called Somatic fusion, is a type of genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both, a somatic hybrid.

3.9 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the gene delivery.
2. Define the electroporation.
3. State the biolistic particle delivery system.

4. Illustrate the sonoporation.
5. What do you understand by the optical transfection?
6. Elaborate on the protoplast fusion.
7. Interpret the retrovirus mediated gene transfer.

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Long-Answer Questions

1. Briefly describe the gene transfer methods in animals.
2. Discuss the gene gun design used for gene delivery.
3. Explain the terms electroporation and microinjection.
4. Define Biolistic approach in detail with advantages and limitations.
5. Analyse the biolistic particle delivery system.
6. Define the sonoporation with the help of example.
7. Illustrate the protoplasm fusion.
8. Discuss the retrovirus mediated gene transfer.

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NOTES

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BLOCK - II
ANIMAL CELL CULTURE AND TRANSGENIC ANIMALS

UNIT 4 CONSTITUENTS OF CULTURE MEDIA

Structure

4.0 INTRODUCTION

Culture medium is a solid, liquid, or semi-solid designed to support the growth of a population of microorganisms or cells via the process of cell proliferation or small plants like the moss Physcomitrella patens. Different types of media are used for growing different types of cells. The two major types of growth media are those used for cell culture, which use specific cell types derived from plants or animals, and those used for microbiological culture, which are used for growing microorganisms such as bacteria or fungi.

Cell culture is one of the major molecular techniques used in life sciences. It involves the removal of cells, tissues or organs from a plant or an animal and its culturing in an artificial environment. Culture media contain all the elements that

most bacteria need for growth and are not selective, so they are used for the general cultivation and maintenance of bacteria kept in laboratory culture collections.

The most critical step of in vitro cell culture is the selection of an appropriate growth medium. By definition, a culture medium/growth medium refers to a liquid or gel produced to maintain the growth and propagation of cells, microorganisms and small plants. All Cell culture media/growth media has a general composition comprising of an appropriate source of energy (carbohydrates) and other compounds which helps in regulate the cell cycle. Generally, a typical culture medium is composed of a glucose (as energy source), amino acids, essential vitamins, inorganic salts, serum (which serves as a source of growth factors), hormones, and attachment factors (aids in the attachment of cells to surface). As mentioned above, growth medium also provides the optimum pH and osmolality for the survival and propagation of cells.

In this unit, you will study about the media requirement, preparation of media, sterilization techniques, natural and synthetic media, culture methods, hanging drop, suspension and monolayer culture, primary and established cell lines.

4.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the media requirement
- Define the preparation of media
- Elaborate on the sterilization techniques
- Analyse the natural and synthetic media
- Explain the culture methods and hanging drop
- Comprehend the suspension and monolayer culture
- Interpret the primary and established cell lines

4.2 CONSTITUENTS OF CULTURE MEDIA

Cell culture media contains a combination of essential nutrients such as glucose, amino acids, salts, vitamins, etc. with each having a specific function. It is obtained from commercial suppliers either as powder or in liquid form. In the next section, we will be discussing the function of each and every component of the cell culture media:

Buffering Systems

The function of the buffering system in the cell culture media is to regulate the pH in order to attain optimum culture conditions for cell growth and division. Two common buffering systems used in cell culture media are:

- **Natural Buffering System:** Cell cultures with a natural buffering system require to be maintained in an air atmosphere having 5-10% CO_2, generally maintained by a CO_2 incubator.

- **HEPES:** HEPES has a finer buffering ability in the pH range of 7.2-7.4 and it does not need a controlled gaseous atmosphere. However, this chemical buffer is highly toxic for certain cell types at higher concentration. Commercially supplied culture media usually include phenol red as a pH indicator, which let constant monitoring of media's pH. Cell culture medium is bright red for pH 7.4, i.e., the optimum pH value for cell culture. At low pH levels, phenol red turns the medium yellow while at higher pH levels it turns the medium purple. However, there are certain disadvantages of using phenol red as pH described like it interferes with flow cytometric studies as well as with sodium-potassium homeostasis.

Inorganic Salt

Inorganic salt in the cell culture media helps to keep the osmotic balance and also help in maintaining the membrane potential by providing essential inorganic salts like sodium, potassium, and calcium ions.

Amino Acids

Amino acids are the building blocks of proteins and are required for the proliferation of cells in a cell culture media. Further, it is essential to supplement the cell culture media with essential amino acids as cells cannot synthesize them on their own. For instance, L-glutamine which is an essential amino acid provides nitrogen for NAD, NADPH and nucleotides, and serves as a secondary energy source for carrying out metabolic activities. Further, cell culture media may be supplemented with nonessential amino acids to stimulate growth and prolongs the viability of the cells.

Carbohydrates

Carbohydrates in the cell culture media are the major source of energy. Most of the cell culture media contains glucose, galactose, maltose or fructose.

Proteins and Peptides

The most commonly used proteins and peptides in cell culture media are albumin, transferrin, and fibronectin. Albumin's binding capacity makes it appropriate for removing toxic substances from the cell culture media. Similarly, Fibronectin act as a key player in cell attachment whereas Transferrin act as an iron transport protein which supplies iron to the cell membrane.

Fatty Acids and Lipids

Fatty acids and lipids are mainly vital in serum-free media.

Vitamins

Cell culture media is supplemented with essential vitamins in sufficient quantity for stimulating growth of cells in culture media.

Trace Elements

Trace elements like zinc, selenium, copper etc. are often supplemented to serum-free media for proper cell growth as well as to maintain the functionality of enzymes.

Antibiotics

Antibiotics are frequently used to control the growth of bacterial, fungal and mycoplasma contamination in cell culture media. However, over-use of antibiotics should be avoided as it can interfere with the metabolism of the cells.

Serum in Media

Most of the commercially supplied cell culture media are supplemented with essential amino acids, carbohydrates, proteins, lipids, vitamins, minerals, hormones, growth factors as well as some trace elements. Serum from fetal and calf bovine sources are regularly used to sustain the growth of cells in culture media. The advantages of using serum free media are as follows:

- Serum provides the basic nutrients required for growth of cells.
- Serum provides several growth factors and hormones necessary for growth and promotion of specialized cell function.
- It can also supplement the media with several binding proteins like albumin, transferrin which acts as transporter for other molecules into the cell. For instance: albumin carries lipids, vitamins, hormones, etc. into the cells.
- It also augments the culture media with proteins like fibronectin which promotes the attachment of cells to the substrate.
- It also augments the cell culture media with spreading factors that help the cells to spread out before they begin to divide.
- It also augments the culture media with protease inhibitors which protect cells from proteolysis.
- It also augments the culture media with trace elements as well as minerals like Na+, K+, Zn^{2+}, Fe^{2+}, etc.
- It augments the viscosity of the culture medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffering agent for the culture media; hence there is no need to add any other buffering agent in the media.

Hence, as seen from above, a medium containing serum contains all the essential factors and nutrients essential for cell growth and propagation. However, serum in cell culture media has some disadvantages like:

1. Serum may contain some growth inhibiting factors.
2. Addition of serum increase the probability of contamination.

3. Addition of serum may interfere with the purification and isolation of cell culture products.

4.3 PREPARATION OF MEDIA

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Commercial suppliers provide cell culture medium in three forms:

- Powdered Form: This type of culture media needs to be prepared and sterilized before use. This is the most economical cell culture media but needs to be filter sterilized before use. Horse sera or fetal bovine sera can be added to the media after filter sterilization.
- Concentrated Form: This type of cell culture media needs to be diluted before use.
- Working Solution: This type of culture media is used directly without any further manipulation.

Irrespective of the cell culture media used for cell growth and propagation, it should be tested rigorously for any fungal, protozoan, bacterial contamination by placing it in a 37°C CO_2 incubator for 72 hours prior to utilization. Cell culture media should always be stored in cool and dark conditions.

4.4 STERILIZATION TECHNIQUES OF MEDIA

Sterilization refers to any process that removes, kills, or deactivates all forms of life (in particular referring to microorganisms such as fungi, bacteria, spores, unicellular eukaryotic organisms such as Plasmodium, etc.) and other biological agents like prions present in a specific surface, object or fluid, for example food or biological culture media. Sterilization can be achieved through various means, including heat, chemicals, irradiation, high pressure, and filtration. Sterilization is distinct from disinfection, sanitization, and pasteurization, in that those methods reduce rather than eliminate all forms of life and biological agents present. After sterilization, an object is referred to as being sterile or aseptic.

4.4.1 Wet Heat Methods (Autoclaving)

This is the most common method of choice for sterilization of media in labs. Autoclaving methodology uses pressurized steam to heat the media or any other material to be sterilized. This is one of the most potent methods that kill all spores, microbes and viruses by hydrolysis and coagulation of cellular proteins, achieved by intense heat in the presence of water. This intense heat comes from the pressurized steam having a very high latent heat when compared to water. This heat is liberated upon contact with the cooler surface of the media or material to be sterilized, letting rapid delivery of heat and fine penetration of dense materials.

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Fig. 4.1 *Parts of an Autclave*

4.4.2 Dry Heat Methods (Baking, Flaming)

Dry heat methods like Flaming and baking are different from wet heat methods like autoclaving as in dry heat methods there's no water, hence protein hydrolysis can't take place. Instead, dry heat methods have a tendency to destroy or kill contaminating microbes by oxidation of cellular components. This requires extra energy than protein hydrolysis so higher temperatures are vital for efficient sterilization by dry heat. For instance, sterilization can be easily achieved in 15 minutes by autoclaving at 121^{o}C while dry heating would normally need a temperature of 160^{o}C to sterilize for the same time frame. However, both wet and dry heat methods are not suitable for carrying out sterilization of culture media containing heat sensitive components.



Fig. 4.2 *Closed View of Hot Air Oven used for Dry Sterilization*

4.4.3 Filtration

Filtration is another technique to rapidly sterilize culture media solutions without wet or dry heating. Filter sterilization technique involves passing the solution through a filter having a pore size such that it allows the culture media to pass through it while preventing the entry of microbes through them. Membrane filters made from cellulose esters are the most common type of filter used for research purpose. Filters with an average pore diameter of 0.2 mm are generally used for removing the bacteria from culture media. However, microscopic contaminants like viruses and phages can easily pass through these filters so filtration is not a fine option if these are a concern.

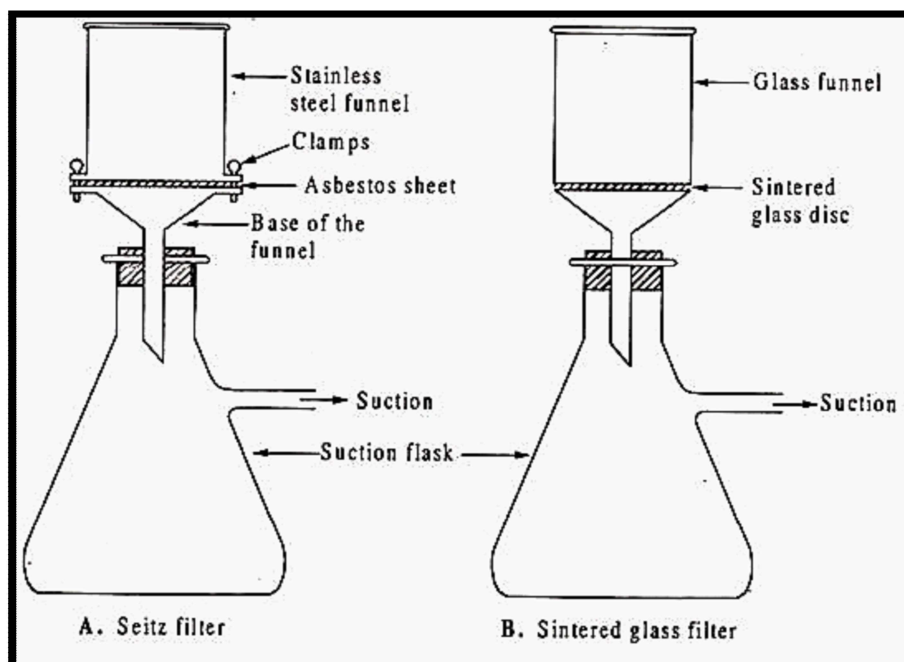


Fig. 4.3 *Filtration Unit used for Sterilization of Culture Media*

4.4.4 Solvents

Ethanol or isopropanol at 60-90% percent concentration (diluted in water) is effective in killing microbial cells however it has no effect on spores. Ethanol or isopropanol functions by denaturing proteins via a method that needs water.

4.4.5 Radiation

UV, X-rays and gamma rays are all types of electromagnetic radiation that have deep destructive effects on DNA, so it makes them outstanding tools for media sterilization.

4.5 NATURAL AND SYNTHETIC MEDIA

NOTES

Natural media comprises of exclusively naturally occurring biological fluids. Natural media are exceptionally valuable and suitable for a broad range of experimental work related to animal cell culture. However, one of the key shortcomings of natural media is its poor reproducibility due to lack of knowledge regarding their exact composition as well as constituents.

Table 4.1 *Table Highlights the Different Types of Natural Media*

	Media Type	Examples
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of the bovine embryo and chick embryo
	Clots	coagulants or plasma clots
	Balanced salt solutions	PBS, DPBS, HBSS, EBSS

Artificial or synthetic media are prepared commercially by adding organic and inorganic nutrients, carbohydrates, serum proteins, essential vitamins, salts, cofactors etc. The advantages of using an artificial media are as follows:

(a) Instant survival of cells after their isolation due to nourishment provided by a balanced salt solution having optimum pH and osmotic pressure).
(b) Lengthened survival of the cells.
(c) Supports indefinite growth.
(d) Helps the cells to perform specialized functions.

All the commercially available artificial media are grouped into four categories:

Serum Containing Media

Most of the commercially supplied cell culture media are supplemented with essential amino acids, carbohydrates, proteins, lipids, vitamins, minerals, hormones, growth factors as well as some trace elements. Serum from fetal and calf bovine sources are regularly used to sustain the growth of cells in culture media. The advantages of using serum containing media are as follows:-

- Serum provides the basic nutrients required for growth of cells.
- Serum provides several growth factors and hormones necessary for growth and promotion of specialized cell function.
- It can also supplement the media with several binding proteins like albumin, transferrin which acts as transporter for other molecules into the cell. For instance, albumin carries lipids, vitamins, hormones, etc. into the cells.

- It also augments the culture media with proteins like fibronectin which promotes the attachment of cells to the substrate.
- It also augments the cell culture media with spreading factors that help the cells to spread out before they begin to divide.
- It also augments the culture media with protease inhibitors which protect cells from proteolysis.
- It also augments the culture media with trace elements as well as minerals like Na+, K+, Zn^{2+}, Fe^{2+}, etc.
- It augments the viscosity of the culture medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffering agent for the culture media; hence there is no need to add any other buffering agent in the media.

Serum-Free Media

As seen from above, a medium containing serum contains all the essential factors and nutrients essential for cell growth and propagation. However, serum in cell culture media has some disadvantages like:

- Serum may contain some growth inhibiting factors.
- Addition of serum increase the probability of contamination.
- Addition of serum may interfere with the purification and isolation of cell culture products.
- It can lead to serious misinterpretations in immunological studies.

A number of serum-free media have been formulated to support the culture of a single cell type, like Knockout Serum Replacement, Knockout DMEM, mTESR1, etc. Serum free media are also referred to as 'Defined Culture Media' as the constituents of these media are known.

Chemically Defined Media

Chemically defined media contain contamination-free ultra-pure organic as well as inorganic constituents and may also contain certain pure protein additives, such as growth factors which provide ample stimulation for cell growth and proliferation. Their ingredients are produced in yeast or bacteria via using genetic engineering with the addition of fatty acids, specific amino acids, essential vitamins, cholesterol, etc.

Protein-Free Media

Protein-free media lack protein and only contain non-protein ingredients. Utilization of protein-free media supports better cell growth, proliferation as well as expression. It also aid in downstream purification of expressed product. Formulation like RPMI-1640 is commercially available protein-free media. Also, they can be supplemented with protein if required.

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4.6 CELL CULTURE METHODS

Cell culture is a multistep process which involves isolation of cells from desirable animal or plant tissues/organs either by mechanical or enzymatic application or they may be derived directly from established cell lines or cell strains followed by their proliferation under optimum conditions in a suitable cell culture media. The three most common cell culture methods are as follows:-

4.6.1 Hanging Drop Method

The classic hanging drop culture is a small drop of liquid, like plasma or some other media permitting tissue growth, suspended from an inverted watch glass. Thereafter, hanging drop is suspended by gravity and surface tension, rather than spreading across a plate. This permits tissues or other cell types to be thoroughly observed without being squashed against a dish. Initially, this methodology was developed to study about bacteria in a confined system. However, 'Ross Granville Harrison' used this technique to show the growth of nerve cells.

Other major applications of this technique comprise stem cells cultures as well as *in vitro* cultures of whole embryos. This technique allows the stem cells to be cultured easily without being pressed against a plate. This is particularly advantageous when the 3D structure of a tissue is preferred. In a similar way, entire embryos can be cultured conveniently by following this technique. For embryology studies, it is essential to maintain the original structure of the embryo, hence hanging drop methodology permits the embryo to develop without getting pressed against a dish. The capability to resolve 3D structures was a significant progress that made this technique a widely used one for research purpose.

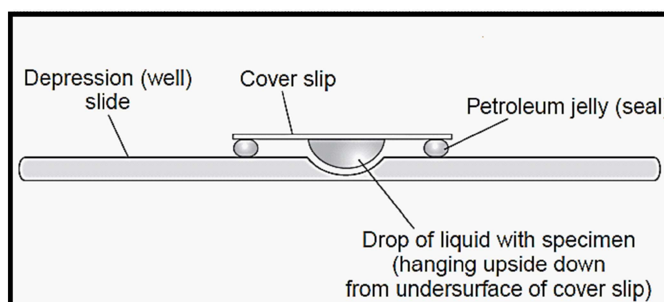


Fig. 4.4 *Principle of Hanging Drops Technique*

4.6.2 Suspension Method

Suspension culture involves multiplication of single cells or cell aggregates when agitated in a liquid medium. Generally, there are two different types of suspension culture, i.e., Batch Culture and Continuous Culture.

(i) Batch Culture

Batch culture is a closed system type of suspension culture where cells undergo proliferation under optimum conditions. Batch cultures are often used to begin

single-cell cultures. A few specific characteristics of Batch cultures are as follows:

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- Cells undergo growth and proliferation in a 200-250 *ml* flask.
- This involves transferring a small amount of suspension culture to a fresh medium.
- The Batch culture follows an S-shaped curve as represented below. The curve is divided into three phases: lag phase where cells adjust to the surroundings and get ready to divide; log or exponential phase where cells undergo proliferation using the growth medium; and finally stationary phase where cells stop dividing after three to four generations of cell division.
- On reaching the stationary phase, it is necessary to transfer an aliquot of cells to a fresh culture medium to revive the cell growth as well as proliferation. If cells existed in the stationary phase for a long time it would lead to the death of the cells.
- During subculturing, researchers allow the culture flask to stand still for a few minutes to settle down the large clumps of tissue.
- After this, a fine syringe is used to take out an aliquot of the suspension from the top most part of the culture.

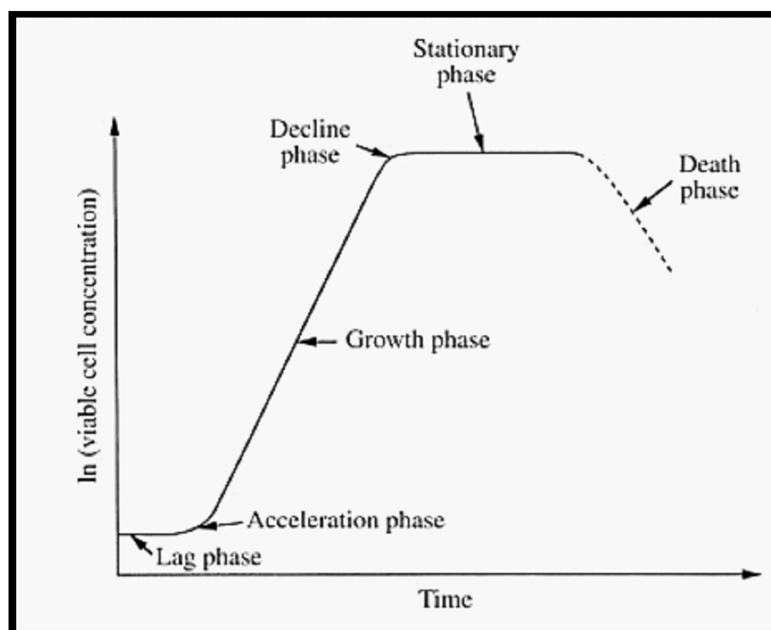


***Fig. 4.5** S-Shaped Curve Showing Different Phases of Cell Growth with Time Duration*

(ii) Continuous Culture

Continuous suspension cultures can either be open or closed system cultures where fresh medium is constantly added and the detoxification of the media is prevented by constantly eliminating leftover nutrients as well as metabolic end products from

the culture medium. A few specific characteristics of the continuous suspension culture are as follows:

- Continuous suspension cultures are specifically appropriate for growing large-scale cultures in the bioreactors.
- In the closed system type of continuous suspension culture, the addition of fresh medium is always balanced by the removal of leftover medium. Hence, cell biomass continues to amplify with the growth and proliferation of the cell.
- In the open system type of continuous suspension culture, medium balance is retained by adding the fresh medium while harvesting the same amount of the culture comprising cell as well as culture medium.

Advantages of Suspension Method

- Suspension method is quite rapid way of cell propagation.
- As seen in the *S*-shaped curve above, the lag phase is relatively shorter.
- Suspension cultures are homogeneous in nature.
- Scaling-up the suspension culture is easy.
- Continuous replacement of the culture medium is not required.
- Suspension cultures are easy to maintain.

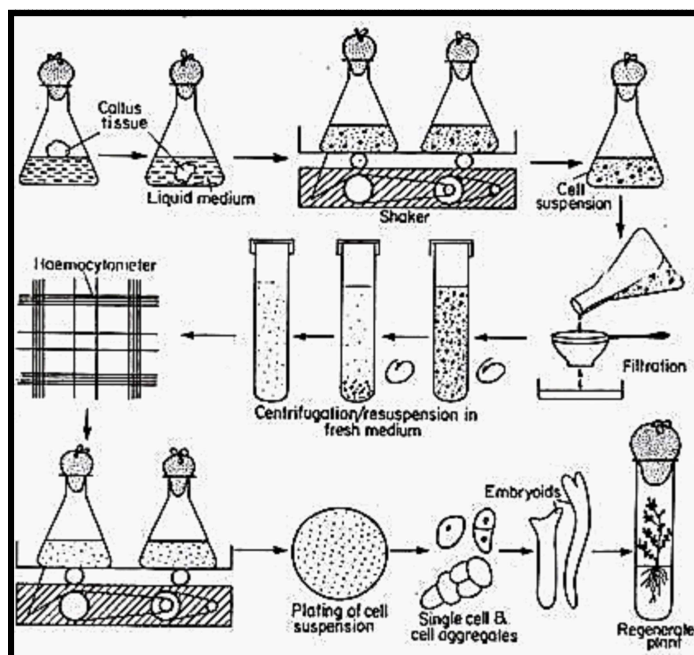


Fig. 4.6 *Method of Cell Suspension Culture and Regeneration of Plant Tissue through Embryogenesis*

4.6.3 Monolayer Culture

Monolayer culture refers to the culture in which bottom of the culture dish is covered with a continuous layer of cells, generally one cell in thickness. The attachment of

cells among themselves as well as to the culture dish is mediated via surface glycoproteins commonly known as cell adhesion molecules. Further, Ca^{2+} ions also served as adhesion molecules.

Mechanical shaking and cell scraping are mechanical methods used for monolayer cultures which are loosely adhered to the dish plate whereas enzymes like trypsin, dispase, pronase, etc. are used for more tightly adhered cultures. Prior to enzymatic dissociation, the monolayer culture are subjected to EDTA for the removal of Ca^{2+} ions which acts as adhesion molecule. In a monolayer culture, sub-culturing is generally carried out somewhere between the middle of the log phase and the time prior to plateau phase. Sub-culturing should never be carried out at lag phase. A good research practice is to subculture monolayer cultures, as soon as they reach confluence. Confluence indicates the culture stage where all the accessible growth area is already utilized by proliferating cells and the cells make close contact with each other. Increase in culture cell density is often accompanied by fall in pH and to maintain the vitality of the culture the medium must be changed followed by subculturing. The process of monolayer subculture involves following steps:

1. Removal of the left over culture medium.
2. Exposing the cells briefly to enzymes like trypsin.
3. Carefully remove the trypsin and dispersion in a culture medium.
4. Incubation of cells to round up.
5. Re-suspension of the cells in a cell culture medium for counting as well as reseeding.
6. Cells reseeded and grown to monolayers.



***Fig. 4.7** Monolayer Culture in a Medium*

4.7 PRIMARY AND ESTABLISHED CELL LINES

Primary Cells line refers to the cells isolated directly from human or animal tissue using mechanical or enzymatic methods. All the isolated cells are placed in a specialized glass or plastic containers having specific tissue culture medium containing all the vital nutrients required for cell growth and division.

Primary cells could be of two types – adherent or suspension. Adherent cells which are usually derived from tissues needs attachment for survival and proliferation are known to be anchorage-dependent cells. While, suspension cells which are usually isolated from blood system do not need attachment for growth and proliferation and are known to be anchorage-independent cells. Even though, primary cells have a limited lifespan, yet they offer huge advantages compared to

established cell lines. For instance, it gives an opportunity to researchers to study factors such as age, sex, race, medical history of the donor while setting up an experiment. It is difficult to attain such tissue complexity and donor variability with established cell lines which are truly systematic and uniform in nature.

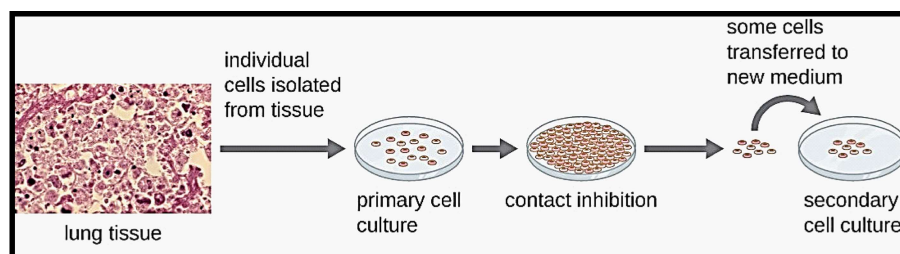


Fig. 4.8 *Primary Cell Culture*

4.7.1 Established Cell Lines

Established cell line refers to the cells that have been constantly passaged over a long period of time due to which they have attained homogenous genotypic and phenotypic characteristics. Established cell lines can be finite cell lines that can be sub-cultured for 20-80 passages after which they undergo senescence or continuous cell lines that has acquired the ability to proliferate indefinitely, either through genetic mutations or artificial modifications. The chief purpose for selecting established cell lines is that they are convenient, easy to handle and widely documented in a lot of research studies published in reputed journals. However, established cell lines are less preferred as a biologically relevant option as they have lost the true characteristics of the original tissue from which they were isolated. Further, serial passaging over a long period of time is reported to cause genotypic and phenotypic variation in cell lines when compared to original cells/tissues thus could result in false positives or negative findings.

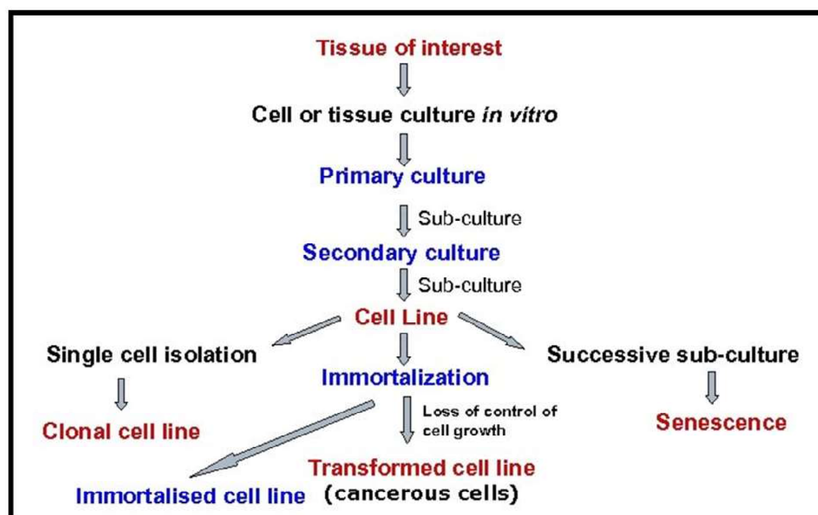


Fig. 4.9 *Production of Established Cell Lines*

Table 4.2 *Table depicts Some of the Established Cell Lines used Regularly for Research Purpose*

Cell line	Species of origin	Tissue of origin	Cell morphology
3T3	Mouse	Connective	Fibroblast
CHO	Chinese Hamster	Ovary	Epithelial
BHK21	Syrian Hamster	Kidney	Fibroblast
HeLa	Human	Cervical Carcinoma	Epithelial
MDCK	Dog	Kidney	Epithelial
MRC-S	Human	Lung	Fibroblast, Finite

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Check Your Progress

1. Explain the term confluence.
2. What are the constituents of a cell culture medium?
3. Define the natural culture media.
4. Interpret the synthetic culture media.
5. Elaborate on the monolayer culture.

4.8 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Confluence indicates the culture stage where all the accessible growth area is already utilized by proliferating cells and the cells make close contact with each other.
2. Generally, a typical culture medium is composed of a glucose (as energy source), amino acids, essential vitamins, inorganic salts, serum (which serves as a source of growth factors), hormones, and attachment factors (aids in the attachment of cells to surface). As mentioned above, growth medium also provides the optimum pH and osmolality for the survival and propagation of cells.
3. Natural media comprises of exclusively naturally occurring biological fluids. Natural media are exceptionally valuable and suitable for a broad range of experimental work related to animal cell culture. However, one of the key shortcomings of natural media is its poor reproducibility due to lack of knowledge regarding their exact composition as well as constituents. A few natural media are plasma, serum, lymph, amniotic fluid, coagulants, extracts of liver, etc.

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4. Artificial or synthetic media are prepared commercially by adding organic and inorganic nutrients, carbohydrates, serum proteins, essential vitamins, salts, and cofactors, etc. Artificial media ensures survival of cells after their isolation due to nourishment provided by a balanced salt solution having optimum pH and osmotic pressure. Artificial media ensures lengthened survival of the cells. Artificial media supports indefinite growth. Artificial media helps the cells to perform specialized functions. For example, RPMI 1640.
5. Monolayer culture refers to the culture in which bottom of the culture dish is covered with a continuous layer of cells, generally one cell in thickness.

4.9 SUMMARY

- Cell culture media contains a combination of essential nutrients such as glucose, amino acids, salts, and vitamins, etc. with each having a specific function. It is obtained from commercial suppliers either as powder or in liquid form.
- HEPES has a finer buffering ability in the pH range of 7.2-7.4 and it does not need a controlled gaseous atmosphere. However, this chemical buffer is highly toxic for certain cell types at higher concentration.
- Sterilization refers to any process that removes, kills, or deactivates all forms of life (in particular referring to microorganisms such as fungi, bacteria, spores, unicellular eukaryotic organisms such as Plasmodium, etc.) and other biological agents like prions present in a specific surface, object or fluid, for example food or biological culture media.
- Dry heat methods like Flaming and baking are different from wet heat methods like autoclaving as in dry heat methods there's no water, hence protein hydrolysis can't take place.
- Filtration is another technique to rapidly sterilize culture media solutions without wet or dry heating. Filter sterilization technique involves passing the solution through a filter having a pore size such that it allows the culture media to pass through it while preventing the entry of microbes through them.
- Natural media comprises of exclusively naturally occurring biological fluids. Natural media are exceptionally valuable and suitable for a broad range of experimental work related to animal cell culture.
- The classic hanging drop culture is a small drop of liquid, like plasma or some other media permitting tissue growth, suspended from an inverted watch glass. Thereafter, hanging drop is suspended by gravity and surface tension, rather than spreading across a plate.
- Monolayer culture refers to the culture in which bottom of the culture dish is covered with a continuous layer of cells, generally one cell in thickness.

- Primary Cells line refers to the cells isolated directly from human or animal tissue using mechanical or enzymatic methods. All the isolated cells are placed in a specialized glass or plastic containers having specific tissue culture medium containing all the vital nutrients required for cell growth and division.
- Established cell line refers to the cells that have been constantly passaged over a long period of time due to which they have attained homogenous genotypic and phenotypic characteristics.

4.10 KEY WORDS

- **Cell culture:** Cell culture media contains a combination of essential nutrients such as glucose, amino acids, salts, and vitamins, etc. with each having a specific function. It is obtained from commercial suppliers either as powder or in liquid form.
- **Sterilization:** Sterilization refers to any process that removes, kills, or deactivates all forms of life (in particular referring to microorganisms such as fungi, bacteria, spores, unicellular eukaryotic organisms such as Plasmodium, etc.) and other biological agents like prions present in a specific surface, object or fluid. For example, food or biological culture media.
- **Filtration:** Filtration is another technique to rapidly sterilize culture media solutions without wet or dry heating. Filter sterilization technique involves passing the solution through a filter having a pore size such that it allows the culture media to pass through it while preventing the entry of microbes through them.
- **Monolayer:** Monolayer culture refers to the culture in which bottom of the culture dish is covered with a continuous layer of cells, generally one cell in thickness.
- **Primary cells line:** Primary Cells line refers to the cells isolated directly from human or animal tissue using mechanical or enzymatic methods.
- **Established cell line:** Established cell line refers to the cells that have been constantly passaged over a long period of time due to which they have attained homogenous genotypic and phenotypic characteristics.

4.11 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Define the term confluence.
2. Explain the constituents of a cell culture medium.

3. State the natural culture media.
4. Illustrate the synthetic culture media.
5. Interpret the monolayer culture.

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Long-Answer Questions

1. Discuss briefly the media requirement and its preparation methods.
2. Explain the sterilization techniques. Give appropriate examples.
3. Differentiate between the natural and synthetic media.
4. Analyse the suspension and monolayer culture.
5. Describe the primary and established cell lines.

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UNIT 5 CHARACTERISTICS OF TRANSFORMED CELLS

Structure

5.0 INTRODUCTION

In molecular biology and genetics, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surroundings through the cell membrane(s). For transformation to take place, the recipient bacterium must be in a state of competence, which might occur in nature as a time-limited response to environmental conditions such as starvation and cell density, and may also be induced in a laboratory.

"Transformation" may also be used to describe the insertion of new genetic material into nonbacterial cells, including animal and plant cells; however, because "Transformation" has a special meaning in relation to animal cells, indicating progression to a cancerous state, the process is usually called "Transfection".

Viral transformation is the change in growth, phenotype, or indefinite reproduction of cells caused by the introduction of inheritable material. Through this process, a virus causes harmful transformations of an in vivo cell or cell culture. The term can also be understood as DNA transfection using a viral vector. Viral transformation can occur both naturally and medically. Natural transformations can include viral cancers, such as Human Papilloma Virus (HPV) and *T*-cell Leukaemia virus type I. Hepatitis *B* and *C* are also the result of natural viral transformation of the host cells. Viral transformation can also be induced for use in medical treatments.

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There are various methods of introducing foreign DNA into a eukaryotic cell: some rely on physical treatment (electroporation, cell squeezing, nanoparticles, magnetofection); others rely on chemical materials or biological particles (viruses) that are used as carriers. Gene delivery is, for example, one of the steps necessary for gene therapy and the genetic modification of crops. There are many different methods of gene delivery developed for various types of cells and tissues, from bacterial to mammalian. Generally, the methods can be divided into two categories: non-viral and viral.

In this unit, you will study about the characteristics of transformed cells, methods of cell preservations, application of cell culture in product development and tissue repair, bioreactors, and scaling-up.

5.1 OBJECTIVES

After going through this unit, you will be able to:

- Define the characteristics of transformed cells
- Explain the methods of cell preservations
- Understand the application of cell culture in product development and tissue repair
- Elaborate on the bioreactors, and scaling-up

5.2 CHARACTERISTICS OF TRANSFORMED CELLS

The process of transformation involves spontaneous or induced permanent phenotypic modifications due to heritable changes in genetic material, i.e., DNA or RNA, hence affecting the gene expression. Transformation of cells may occur due to any one of the following reasons:

- Transformation may occur spontaneously
- Transformation may occur due to infection with transforming virus.
- Transformation may occur from gene transfection.
- Transformation may occur due to exposure to radiations or carcinogenic agents

The four characteristics features of transformed cells are as follows:

(1) Genetic Instability

(2) Immortalization

(3) Aberrant Growth Control

(4) Tumorigenicity

In the next section, we will be going to discuss the characteristics features of transformed cells:

(1) Genetic Instability

Generally, cell lines in culture are prone to genetic variability or instability. Genetic variations in the cultured cells occurs either due to high rate of spontaneous mutations owing to high rate of cell proliferation or due to constant existence of mutant cells in the culture medium, as they are not eliminated.

(2) Immortalization

The attainment of an infinite life span by a cell is known as immortalization. Normally cells have a finite life span of 20-100 generations; however, most of the tumour cells which produce continuous cell lines have infinite life span. It has been observed that finite life span of cultured cells is regulated by nearly ten dominantly acting senescence genes whose products restrains or slows down the progression of cell cycle. Immortalization in cells occurs due to inactivation of cell cycle regulatory genes such as p53 genes, and Rb, etc.

To achieve the process of immortalization or in other words to extend the life span of cell, cells are infected with retroviruses containing immortalizing genes before they enter senescence. Subsequently, the cells cease to divide, and enter an emergency stage that go on for some months at stretch. Finally, a fraction of cells can grow, and ultimately developed into immortalized cells.

(3) Aberrant Growth Control

The transformed cells exhibit several aberrations with respect to growth characteristics such as:

(a) Anchorage Independence

The alterations on the surface of transformed cells such as modifications in the cell surface glycoproteins and integrin's or loss of fibronectin or loss/lack of Cell Adhesion Molecules (CAMs) leads to reduction in cell—cell or cell- substrate adhesion. These results in loss of cell's requirement for attachment as well as spreading to proliferate, a phenomenon referred to as anchorage independence. Anchorage independent cells often grow in a haphazard or disorganized way.

(b) Contact Inhibition

The transformed cells are also characterized by a specific feature known as loss of contact inhibition. This often results in a reduced density constraint of growth, followed by higher saturation density when compared to normal cells. This can be easily observed by the morphological changes in the transformed cells such as disoriented and disorganized monolayer cells.

(c) Low Serum Requirement

Transformed cells secretes autocrine growth factors like Colony Stimulating Factor (CSF), Transforming Growth Factor (TGFa), and Vasoactive Intestinal Peptide

(VIP), etc., in huge quantity when compared to normal cells and hence does not require serum for their growth as well as proliferation.

(4) Tumorigenicity

Cell transformation is a multifaceted process resulting in the development of neoplastic cells. The cell lines acquired from malignant tumours are already transformed. The cell lines attain tumorigenicity in the in vitro culture due to uncontrolled growth rate and proliferation, immortalization as well as reduced anchorage dependence.

5.3 METHODS OF CELL PRESERVATIONS

Cell preservation refers to the entire process of extraction, processing and storage of cells so that they can be used in the future for research, scientific or medical purpose. Cell preservation techniques aim at maintaining healthy cell cultures by providing them an environment which helps the cells to retain their specific properties. Although, it is a difficult task to achieve as cell cultures can easily become contaminated which renders them useless. Further, preservation techniques applies very high or low temperatures (above about 600^{o}C and below -200^{o}C) that initiates the process of denaturation of proteins in cells leading to cell death. Hence, it is essential to apply a suitable cell preservation technique to prevent the loss of cells. Cell preservation techniques vary according to the type and age of cells. There are two primary types of cell preservation namely: - cryopreservation and hypothermic preservation. All the other cell preservation methods have been derived from these two primary types of cell preservation. Hypothermic preservation works only for a couple of hours in a clinical setting with the anticipation of transplantation whereas cryopreservation is for long term storage, up to years. In the next section, a few popular cells preservation techniques have been discussed.

5.3.1 Hypothermic Preservation

Hypothermic preservation is not used primarily for cell preservation but suitable for tissue and organ preservation as well as for transportation where the time period is short. Hypothermic mode of cell preservation has several advantages like decrease in metabolic rate and oxygen demand whereas the negative effect of this preservation is protein denaturation. To reduce the negative effects of hypothermic preservation, cell preserving solutions such as non-electrolytes (raffinose, sucrose, saccharides), citrate and magnesium chelates are used frequently to prevent intracellular edema whereas buffers and mannitol are used to deal with acidosis, and free radical production, etc.

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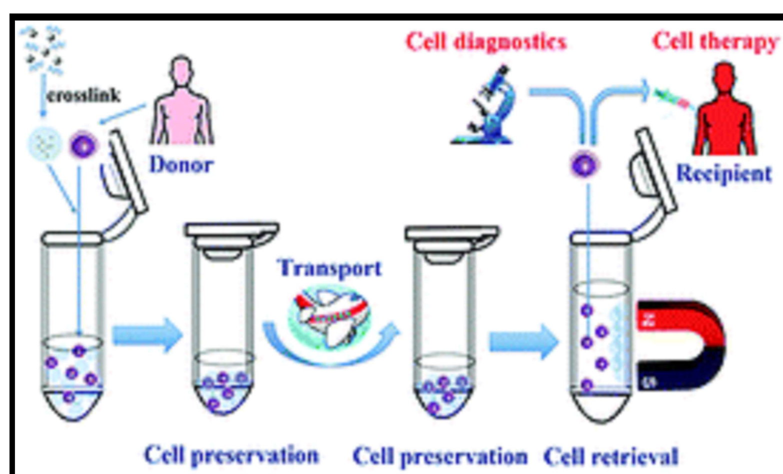


Fig. 5.1 *Hypothermic Mode of Cell Preservation*

5.3.2 Cryopreservation

Cryopreservation is generally carried out at low temperatures to slow down cell's biological activity or in other words to let cells continue in the nearly animated state where there is little or no biological activity. Let us discuss the entire process of cryopreservation step by step:-

Collection and preservation of Cells and Tissues

Generally, thin samples of tissues or clump of individual cells like semen, blood, stem cells, eggs, and plant material (seed or shoot) can be cryopreserved conveniently.

Addition of Cryoprotectant

During cryopreservation cell can damage either due to formation of large ice crystals inside the cell or when intracellular concentration of solutes increases to toxic levels before or during freezing as a result of dehydration. Now, damage to cells and tissues can be achieved by two techniques:-

(1) **Vitrification-** Here, the ice formation cannot take place at all as the aqueous solution is highly concentrated. Instead, water gets solidified into amorphous glassy state.

(2) **Cryoprotective Dehydration-** Dehydration increases the osmotic pressure of the intracellular contents which lowers down its freezing temperature.

Cryoprotectant acts like antifreeze, i.e., they lower down the freezing temperature of the material to be cryopreserved and increases its viscosity. Cryoprotectants like glycerol, mannitol, and propylene, etc. can be used effectively to prevent the damage to cell and its intracellular structures.

Freezing- It can be achieved by:

(1) Slow Freezing Method- The tissue is frozen slowly and then transferred to liquid nitrogen.

(2) Rapid Freezing Method-The tissue is plunged directly into liquid nitrogen

Storage- Storage of the material to be cryopreserved is as essential as freezing. Usually, the material is stored at a range from -70° C to -196° C. The main goal of the storage is to stop all the metabolic and biological activities happening inside the cell.

Thawing- Thawing is done by keeping the aliquot containing the cryopreserved material in a water bath having a temperature of 35° C to 45° C.

Benefits of Cryopreservation

(1) This technique is highly useful in the breeding of dairy cattle, dogs, and pigs.

(2) The cryopreserved material can be stored easily for several years.

(3) Stem cells, skin cells, umbilical cord cells can be preserved by this technique for a long duration of time and can be used whenever required for transplantation or research purpose.

(4) Cancer and tumor cells can be preserved by this technique for future research.

Disadvantages of Cryopreservation

(1) A lot of social and ethical issues are associated with it.

(2) High cost of the process.

The entire process of cryopreservation can be summarized as follows:

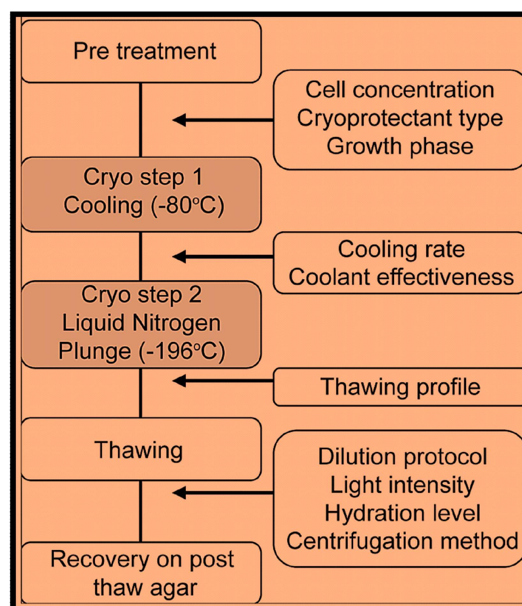


***Fig. 5.2** Mechanism of Cryopreservation*

5.4 APPLICATIONS OF CELL CULTURE IN PRODUCT DEVELOPMENT AND TISSUE REPAIR

Cell culture provides outstanding model systems for carrying out the research on normal physiology and biochemistry of cells involving concepts like metabolic studies, aging, mutagenesis, carcinogenesis, and also the effects of drugs and other toxic compounds on the cells. Besides this, it also helps in drug screening as well as development and production of active biological compounds like vaccines and therapeutic proteins in large quantity. The applications of cell culture system are as follows:

Cell Culture as Model System

- Cell culture are used regularly as model system to study the concepts of basic cell biology as well as biochemistry.
- To study the interaction between cell and pathogen like bacteria, viruses, etc.
- To carry out the drug screening.
- To carry out the research on metabolic processes like ageing.
- Cell culture system can be used to carry out the cancer research, i.e., it can help the researchers to reveal the mechanism behind the conversion of normal functioning cells to cancerous.
- Further, it can also help to screen the drugs that can be effectively used to destroy cancer cells.

Cell Culture to Study Virology

- Animal cell culture system can be used to replicate the viruses as an alternative to animals for the mass production of vaccine.
- Cell culture system can also be used to identify and isolate viruses as well as to study growth and development cycle of viruses.
- Cell culture system can also be used to study the mode of infection used by different viruses.

Cell Culture System in Toxicity Testing

- Animal cell culture system can be used to study the effects of newly discovered drugs, cosmetics as well as chemicals on survival, growth and development of different cell types.
- Cell culture system can also be used to determine the maximum permissible safe dosage of newly discovered drugs.

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Cell Culture System in Vaccine Production

- Cell culture system can be used in the production of viruses and these viruses can be used to produce vaccines for diseases.

Cell Culture System in Production of Genetically Engineered Protein

- Cell culture system can be used to manufacture genetically engineered biological compounds such as insulin, hormones, and monoclonal antibodies, etc.

Cell Culture System as Substitute for Vital Tissue or Organs

- Cell culture system can be used as a substitute for vital tissues as well as organs. For instance, skin produced by cell culture system can be used to treat patients having severe burns and ulcers.
- Advance researches are going in the direction to produce organs like kidney, liver, and pancreas, etc.
- Advance researches are going on in the field of organ culture techniques on both embryonic as well as adult stem cell culture. These cells are pluripotent, i.e., they have the unique ability to differentiate into several different types of cells as well as organs.

Cell Culture System in Genetic Counseling

- Fetal cell cultures extracted from pregnant women are generally used to study or examine the abnormalities of chromosomes.
- Such culture can help in determining the chromosomal abnormalities as well as in the detection of fetal disorders.

Cell Culture System in Genetic Engineering

- Cell culture system can be used to develop transgenic cells, i.e., cells having new genetic material like DNA or RNA.
- Such transgenic cells can be used to study the expression of new genes as well as its effect on cell.
- Such cell lines can be developed commercially to extract the desirable protein product.
- Further, such genetically altered cells can be used in gene therapy techniques.
- Such genetically altered cells having the desirable functional gene can be introduced into the patient lacking or missing that functional gene.

5.5 BIOREACTORS

A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH

level, oxygen levels and stirring rate to yield the maximum cell growth and productivity. Bioreactors are generally used for making pharmaceutical products such as antibiotics and insulin in large quantities.

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Features of Bioreactor

- Bioreactors are basically systems or devices that supports a biologically active environment.
- They are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of stainless steel.
- Inside the Bioreactor, chemical or biochemical process is carried out under homogenous environment by constantly stirring the contents.
- Bioreactor ensures the *controlled* environment by maintaining the same temperature, pH, as well as oxygen levels.
- The reaction carried out inside a bioreactor can be either aerobic or anaerobic.

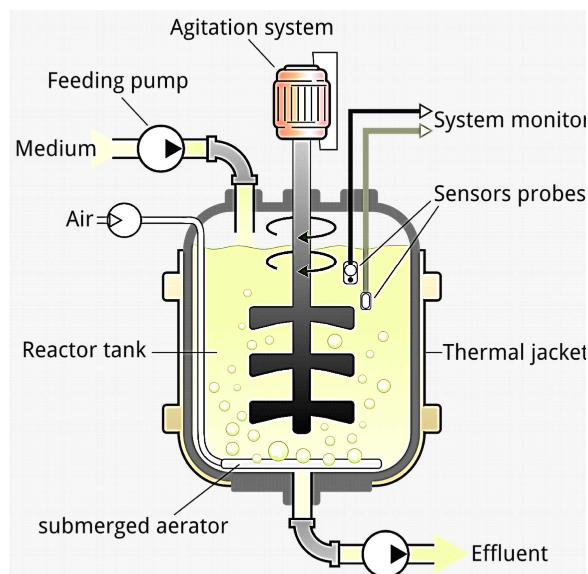


Fig. 5.3 *Bioreactor*

5.5.1 Stages in a Bio-Process

The three main stages to a bio-process consist of: - upstream processing, bioreaction and downstream processing. In the next section, we will discuss each stage in detail:-

(1) *Upstream Processing*

In this step, raw material isolated from a biological or non-biological origin is transformed to a more suitable form for processing. This stage involves the following process

(i) Chemical hydrolysis, (ii) Preparation of liquid medium, (iii) Parting of particulate, (iv) Air purification

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(2) *Bioreaction*

Next stage, i.e., the bioreaction stage consists of three operations namely:

(i) Production of biomass

(ii) Metabolise biosynthesis

(iii) Biotransformation

(3) *Downstream Processing*

This is the final stage where the material is further transformed to a more useful form. This stage consists of five operations namely:-

(i) Solid liquid separation-This involves separation of cells from broth or culture media. Some of the techniques involved in the solid-liquid separation are centrifugation, filtration and flocculation.

(ii) Adsorption

(iii) Liquid-liquid extraction

(iv) Distillation

(v) Drying

All the steps mentioned above have been discussed in detail in the previous unit as well as in the next section.

5.5.2 Types of Bioreactor Processes

There are three main types of bioreactor processes which are used these are, batch, continuous, and fed-batch.

(1) *Batch Bioreactor Processes*

Batch bioreactor progressions involve filling the bioreactor with substantial amount of medium as well as the inoculums and working the bioreactor with no additional nutrients or medium until after the growth profile is complete.

(2) *Continuous Bioreactor Processes*

Continuous bioreactor progressions involve the bioreactor being constantly fed with required nutrients as well as the medium, as the reactor is persistently harvesting material. The harvested material is being collected continually resulting in larger volumes of harvested material and longer bioreactor campaigns. However, the only disadvantage associated with such long processes is that it drastically increases the chances of contamination.

(3) *Fed-Batch Bioreactor Processes*

Fed-batch bioreactor progressions are the most common reactor processes used in the commercial setup. This methodology begins with a low starting volume and feeds nutrients as well as medium on a planned schedule without removing the harvest material. The entire harvest material is collected after the process is complete and then processed for downstream processing.

5.6 SCALE UP PROCESS

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Scale-up process involves the progression of culture systems from small scale laboratory production to large scale industrial or commercial production. This methodology aims at increasing the scale of a culture depending upon the proliferation of cells. All the scale up process can be broadly divided into two categories namely:

- **Scale-up in suspension**-Scale-up of suspension culture chiefly involves an increase in the volume of the culture.
- **Scale-up in monolayer-** The monolayer cultures are anchorage- dependent culture. This process involves increase in the surface area of the substrate in proportion to the number of cells as well as volume of the medium.

There are many different types of bioreactors like: stirred-tank, rocker, air lift, and fixed-bed, etc. which are designed specifically to scale up the process. In the next section, we will discuss different types of bioreactors used for scaling up process one by one:

1. Stirred-Tank Bioreactors

Stirred-Tank Reactors (STRs) are the most widely-used bioreactors and are well equipped with an **impeller** for homogenizing the culture media as well as a **sparger** for delivering the oxygen to the cells. STRs vary in size from 15 mL to 2000 L for single-use in stainless-steel body. STRs are chiefly used to scale-up a procedure from research and development scale or laboratory scale to manufacturing or industrial scale. The major aim is to ensure that a process at a smaller volume can act as a representative of larger volumes.

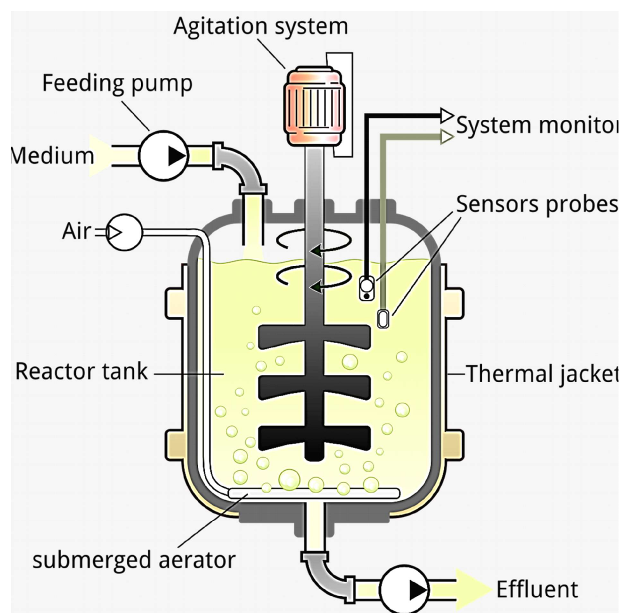


Fig. 5.4 *Continuous Stirred-Tank Type Bioreactor*

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2. Rocker Bioreactors

Rocker bioreactors are offered as single-use systems. They comprises of a bag on a moving platform, relying on a rocking motion for the mixing. Oxygen diffuses takes place through the headspace via liquid-gas interface, as rocket bioreactors lacks sparger which is used for delivering oxygen to the cells. Rocket bioreactors vary in size from a few litres to a maximum of 100 L. They are primarily used for small-scale production or for seeding into larger bioreactors.

3. Air Lift Bioreactors

Air lift bioreactors are not very often used in the biopharmaceuticals industry as they are not very popular with regulatory processes that largely remained unexplored. Air lift bioreactors rely on air bubbles to aerate as well as to carry the media around the reactor for mixing at the same time.

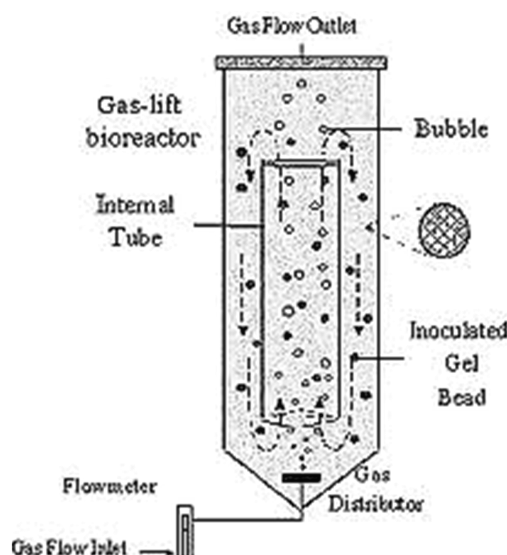


Fig. 5.5 *Simplistic View of Air Lift Bioreactors*

4. Fixed-Bed Bioreactors

Fixed-bed bioreactors are used for adherent-cells i.e. the cell types that can only grow only when they are attached to a surface. Fixed-bed bioreactors usually have strips of fibers (carriers) which are packed together to make the fixed-bed. These carriers ensure a high surface area for the cells to adhere to. Aerated culture medium flows through it. Further, anchorage dependent cells can also be cultivated in other bioreactors such as stirred-tank, rocker and air lift bioreactors just like the suspended cells. However, they require to be attached to 100-micrometre beads (i.e. microcarriers) placed in suspension. Scale-up of this process is a tedious, laborious and time-consuming process when compared to suspension process or the adherent cells process in the desirable fixed-bed bioreactors.

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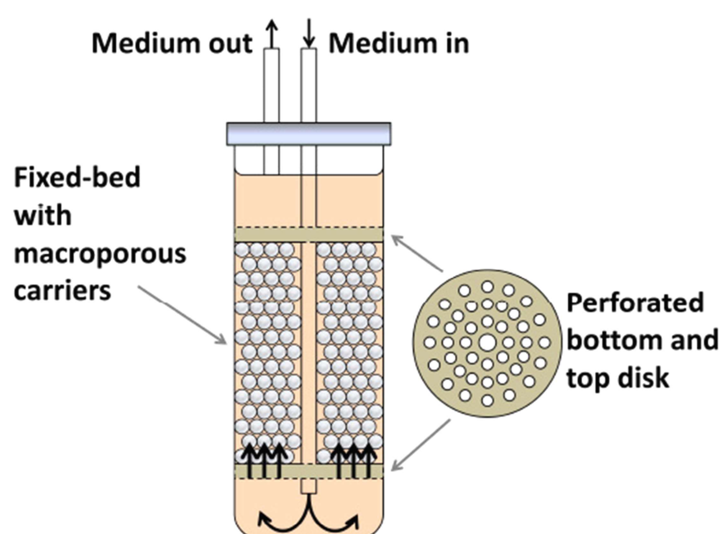


Fig. 5.6 Simplistic View of a Fixed Bed Unit

Check Your Progress

1. Elaborate on the bioreactors.
2. Define the cell culture.
3. What is the use of scale up process?
4. Explain the term cell preservation.
5. Illustrate the culture media.

5.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity. Bioreactors are generally used for making pharmaceutical products such as antibiotics and insulin in large quantities.
2. Cell culture involves the removal of cells, tissues or organs from a plant or an animal and its culturing in an artificial environment. Cell requires optimal conditions for their survival and proliferation like:- A substrate for cell attachment, an appropriate growth medium/nutrient media that provides correct pH as well as osmolality and an incubator that maintains controlled temperature, pressure, and humidity, etc.
3. Scale-up process involves the progression of culture systems from small scale laboratory production to large scale industrial or commercial production.

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4. Cell preservation refers to the entire process of extraction, processing and storage of cells so that they can be used in the future for research, scientific or medical purpose. Cell preservation techniques aim at maintaining healthy cell cultures by providing them an environment which helps the cells to retain their specific properties.
5. All Cell culture media/growth media has a general composition comprising of an appropriate source of energy (carbohydrates) and other compounds which helps in regulate the cell cycle. Generally, a typical culture medium is composed of a glucose (as energy source), amino acids, essential vitamins, inorganic salts, serum (which serves as a source of growth factors), hormones, and attachment factors (aids in the attachment of cells to surface).

5.8 SUMMARY

- The process of transformation involves spontaneous or induced permanent phenotypic modifications due to heritable changes in genetic material, i.e., DNA or RNA, hence affecting the gene expression.
- Cell preservation refers to the entire process of extraction, processing and storage of cells so that they can be used in the future for research, scientific or medical purpose. Cell preservation techniques aim at maintaining healthy cell cultures by providing them an environment which helps the cells to retain their specific properties.
- Cell preservation techniques vary according to the type and age of cells. There are two primary types of cell preservation namely: - cryopreservation and hypothermic preservation. All the other cell preservation methods have been derived from these two primary types of cell preservation.
- Hypothermic preservation is not used primarily for cell preservation but suitable for tissue and organ preservation as well as for transportation where the time period is short.
- Cryopreservation is generally carried out at low temperatures to slow down cell's biological activity or in other words to let cells continue in the nearly animated state where there is little or no biological activity.
- A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity.
- Scale-up process involves the progression of culture systems from small scale laboratory production to large scale industrial or commercial production. This methodology aims at increasing the scale of a culture depending upon the proliferation of cells.

5.9 KEY WORDS

- **Cell preservation:** Cell preservation refers to the entire process of extraction, processing and storage of cells so that they can be used in the future for research, scientific or medical purpose.
- **Hypothermic preservation:** Hypothermic preservation is not used primarily for cell preservation but suitable for tissue and organ preservation as well as for transportation where the time period is short.
- **Cryopreservation:** Cryopreservation is generally carried out at low temperatures to slow down cell's biological activity or in other words to let cells continue in the nearly animated state where there is little or no biological activity.
- **Bioreactor:** A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity.
- **Scale-up process:** Scale-up process involves the progression of culture systems from small scale laboratory production to large scale industrial or commercial production. This methodology aims at increasing the scale of a culture depending upon the proliferation of cells.

5.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the bioreactors.
2. Illustrate the cell culture.
3. What is the use of scale up process?
4. Elaborate on the cell preservation.
5. Interpret the culture media.

Long-Answer Questions

1. Discuss briefly the characteristics of transformed cells.
2. Explain the methods of cell preservation with the help of examples.
3. Analyse the application of cell culture in product development and tissue repair.
4. Describe the bioreactors with its applications.
5. Illustrate the scaling-up technologies.

5.11 FURTHER READINGS

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UNIT 6 PRODUCTION AND APPLICATIONS OF TRANSGENIC ANIMALS

Structure

6.0 INTRODUCTION

Genetically modified animals are animals that have been genetically modified for a variety of purposes including producing drugs, enhancing yields, increasing resistance to disease, etc. The vast majority of genetically modified animals are at the research stage while the number close to entering the market remains small.

Genetically modified mammals are mammals that have been genetically engineered. They are an important category of genetically modified organisms. The majority of research involving genetically modified mammals involves mice with attempts to produce knockout animals in other mammalian species limited by the inability to derive and stably culture embryonic stem cells.

A transgene is a gene that has been transferred naturally, or by any of a number of genetic engineering techniques from one organism to another. The introduction of a transgene, in a process known as transgenesis, has the potential to change the phenotype of an organism. Transgene describes a segment of DNA containing a gene sequence that has been isolated from one organism and is introduced into a different organism. This non-native segment of DNA may

either retain the ability to produce RNA or protein in the transgenic organism or alter the normal function of the transgenic organism's genetic code. In general, the DNA is incorporated into the organism's germ line. For example, in higher vertebrates this can be accomplished by injecting the foreign DNA into the nucleus of a fertilized ovum. This technique is routinely used to introduce human disease genes or other genes of interest into strains of laboratory mice to study the function or pathology involved with that particular gene.

In this unit, you will study about the production and applications of transgenic animals (Fish, Mice, Sheep, and Pig), chimeras, and the Dolly.

6.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the production and applications of transgenic animals (Fish, Mice, Sheep, and Pig)
- Elaborate on the chimeras
- Explain about the Dolly (first clone produced from a cell taken from an adult mammal)

6.2 PRODUCTION OF TRANSGENIC ANIMALS

The foundation for the production of transgenic animals began using sperm mediated gene transfer and in 1980s, the transgenic mice are produced via using the most accepted microinjection technique. Subsequently, production of several transgenic animals was reported in 1985.

There are several types of transgenic animals like transgenic chickens, sheep, pigs, birds, and insects, etc. Microinjection was one of the widely accepted and frequently methodology used for the process of transgenics, however, it has some major drawbacks like low competence, and inconsistent expression patterns, etc. Hence, researchers began to look for alternative methods like intracytoplasmic injection of sperm heads carrying foreign DNA, sperm-mediated DNA transfer, injection or infection of oocytes or via different types of viral vectors, RiboNucleic Acid (RNA) interference technology as well as the use of nuclear transfer technology.

Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding. The steps involved in creating a transgenic organism are as follows:

1. Identification of the Desirable Gene

The first step is the identification of the desirable gene or gene of interest that codes for a specific target protein. A gene that codes for a desirable trait or protein

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must first be identified. Several molecular techniques like Gene Chips (Microarray) and DNA sequencing can be used to identify the desirable gene.

2. Isolation of Desirable Gene

The second step involves the isolation of the desirable gene from the target species. It can be achieved either via mechanically breaking the cells or with the aid of chemical agents like detergents. The entire DNA can be then separated from the other cell components via technique known as cell centrifugation. Now, to separate the target gene from the total DNA content following steps needs to be followed:

- Separation of DNA fragments according to size via Gel Electrophoresis.
- Identification of the gene of interest using a DNA probe.
- Cut out of the gel and amplified (copied) using PCR.
- Alternatively, gene of interest could be inserted into a bacterial plasmid using the enzyme DNA Ligase.
- Bacteria would automatically copy the gene while undergoing cell division- a technique popularly called as Gene Cloning.
- However, if enough information is available regarding the gene of interest then it might be possible to create specific DNA primers and copy the gene using PCR without isolating it on a gel.

3. Transformation of the Desirable Gene

- Finally, a vector (varies according to cell type) is used to transfer the gene of interest into the organism being modified.
- The final DNA sequence that is prepared consisting of target gene and associated regulatory sequences (promoter and termination) sequences is referred to as Gene Construct.
- However, the success rate at which transgene is expressed is very low.
- For the target gene to be expressed, it must make its way into the nucleus.
- For it to be passed on during cell division (mitosis and meiosis) it must integrate itself into the target cells genome via recombination /crossing over.
- For verifying, whether, the target gene has been inserted into the genome or not- researchers incorporate a second gene known as reporter gene into the gene construct. This second gene codes for an easily selectable / observable characteristic like antibiotic resistance or glow in the dark protein.
- This enables the researchers to easily verify whether the integrated gene is expressing or not.

Though, there are several social, extrinsic, and intrinsic ethical issues as well as legal implications are associated with the transgenic animals. However, transgenic animals have several potential use:

- Transgenic animals are widely used as disease model.
- Transgenic animals as food.

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- Transgenic animals in drug and industrial production.
- Transgenic animals in disease control.
- Transgenic animals in xenotransplantation.
- Transgenic combinations might help researchers to develop vaccines against deadly diseases.
- Transgenic animals in blood replacement.
- Transgenic animals are used in toxicity testing.
- Transgenic animals in agriculture.

6.2.1 Methods of Producing Transgenic Animals

The basic principle in the production of transgenic animals is the introduction of a foreign gene or genes into an animal. The new inserted foreign gene is referred to as the transgene.

6.2.2 DNA Microinjection

This method involves the direct microinjection of the desirable gene construct (either a single gene or a grouping of desirable genes) from another individual of the same species or from an entirely different species, into the pronuclear of a fertilized ovum. Microinjection is the most widely used and accepted methodology in mammals as it can be easily applied to a variety of species. However, the newly introduced foreign gene may over- or under-express depending upon the insertion of gene which is a highly random process. The manipulated fertilized ovum is then transferred into the oviduct of a foster mother that has been prepared to act as a recipient by mating with a vasectomized male.

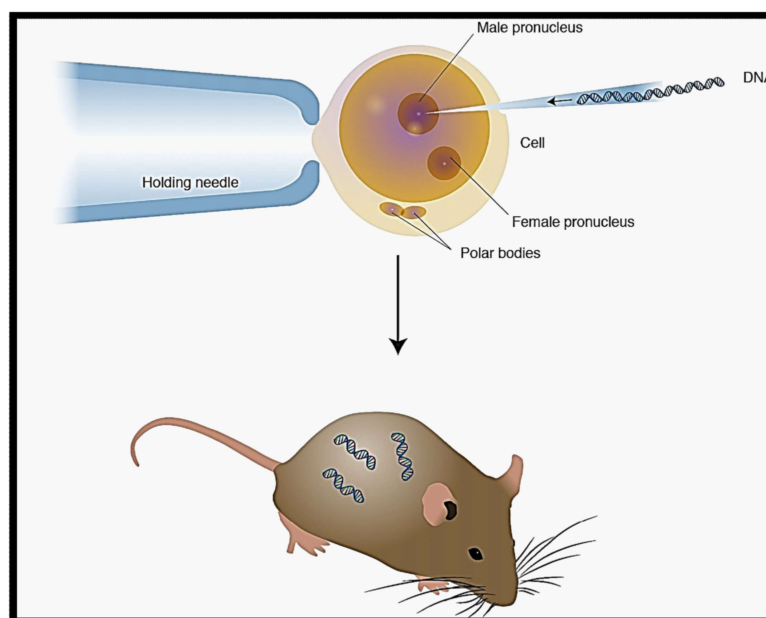


Fig. 6.1 *Production of Transgenic Mice by the Method of DNA Microinjection*

6.2.3 Embryonic Stem Cell-Mediated Gene Transfer

1. Stem cells are undifferentiated cells that have the potency to differentiate into different type of cell (somatic and germ cells) and therefore to give rise to a complete organism. The following are the properties of **Embryonic Stem Cells (ESCs):**
   - They are derived from early stages of embryo development i.e. from mouse or human blastocyst.
   - They are pluripotent, self-renewing cells.
   - They can be stored in culture for a long duration of time as undifferentiated cell lines.
   - These undifferentiated cells can be stimulated to differentiate into any cell line.
   - ESCs can differentiate into endoderm, mesoderm, and ectoderm embryonic germ layers or any type of somatic cells.
   - They are of wide significance in tissue regeneration therapy.
   - Embryonic stem cells are also derived in-vitro as shown in the Figure 6.2 below:

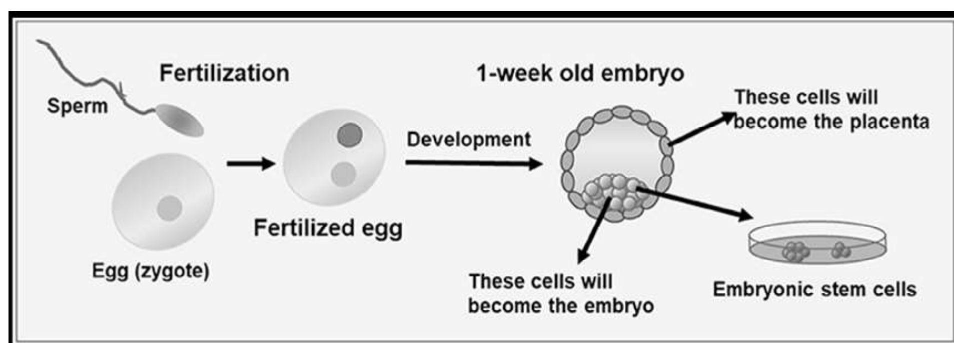


***Fig. 6.2** In-Vitro Isolation of Embryonic Stem Cells*

This method involves insertion of the desirable foreign DNA via homologous recombination into an in vitro culture of Embryonic Stem (ES) cells. These cells are then allowed to develop in tissue culture media and incorporated into an embryo at the blastocyst stage of development. This results in a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method. This methodology is of utmost importance for the study of the genetic control of developmental processes. This methodology has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

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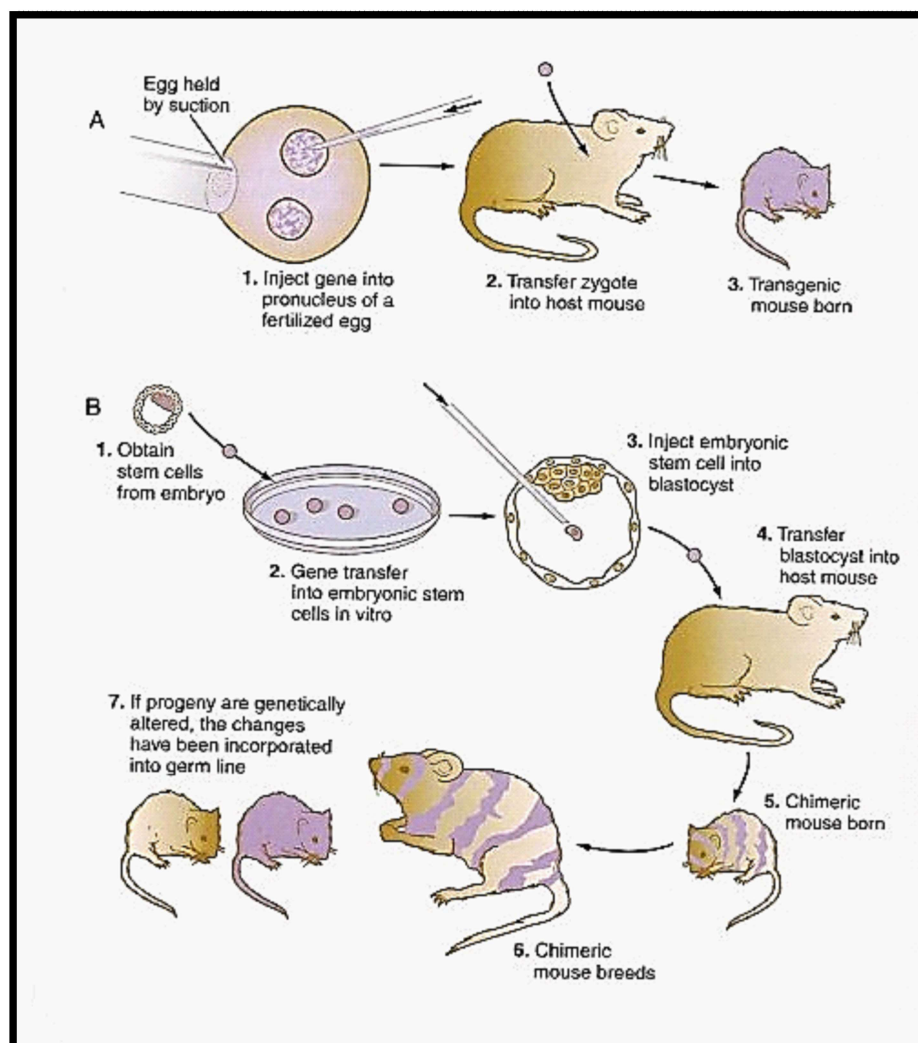


Fig. 6.3 *(A) - DNA Microinjection Method of Creation of Transgenic Mice; (B) - Embryonic Stem Cell Mediated Gene Transfer Method of Producing Transgenic Mice*

6.2.4 Retrovirus-Mediated Gene Transfer

Retrovirus-mediated gene transfer is mediated by means of a carrier or vector, normally a virus or a plasmid. Retroviruses are the most common choice to be used as vectors to transfer genetic material (DNA or RNA) into the cell, taking benefit of their capability to infect host cells in this way. Offspring resulting from this technique are chimeric animals, i.e., not all of them carrying the retrovirus. Transmission of the foreign gene is achievable only if the retrovirus incorporates into some of the germ cells.

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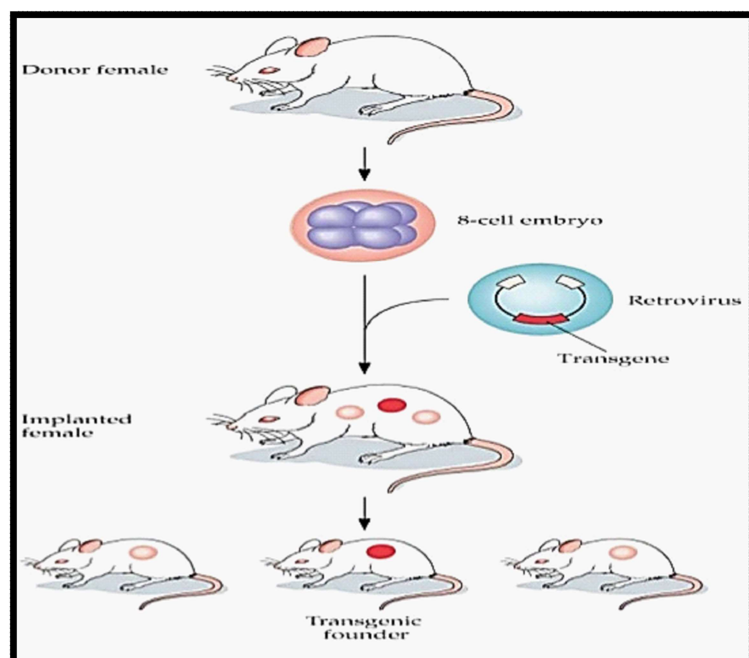


Fig. 6.4 Retrovirus-Mediated Gene Transfer Method of Producing Transgenic Animals

6.2.5 Somatic Cell Nuclear Transfer Technology

The details of technique are discussed below:

- Firstly, the nucleus of a somatic (body) cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed).
- Secondly, the somatic nucleus present inside the egg, is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg).
- Thirdly, zygote is stimulated to undergo division divide by an electric shock.
- Lastly, when the developing zygote forms a blastocyst, it is implanted in the womb of a surrogate mother.

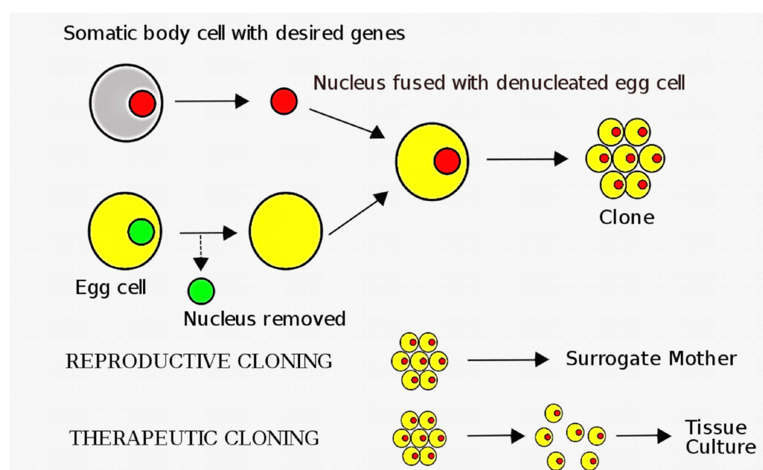


Fig. 6.5 Somatic Cell Nuclear Transfer Technology of Producing Transgenic Animals

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However, for any of the above discussed techniques the success rate in terms of live birth of animals containing the transgene is extremely low.

6.3 APPLICATIONS OF TRANSGENIC ANIMALS

- Transgenic Animals are widely used as Disease Model: Historically, mice have been used to model human disease because of their physiological, anatomical and genomic similarities to humans. Transgenic animals such as mice are quite popular with researchers to study critical diseases such as Alzheimer's, cancer, and AIDS. Transgenic animals enable scientists to be aware of the role of genes in specific diseases.
- Transgenic Animals as Food: The FDA suggested that cloned animals and their products are edible.
- Transgenic Animals in Drug and Industrial Production: Transgenic animals are routinely used for production of therapeutic proteins used in the treatment of diseases such as emphysema or cystic fibrosis. Industries are investing a lot of money in research and development to derive therapeutic proteins like monoclonal antibodies from the milk of transgenic animals such as rabbits, goats and cows to administer drugs in treatment protocols for disorders such as cancer, rheumatoid arthritis and other autoimmune disorders.
- Transgenic Animals in Disease Control: Researchers developed the mice by altering the genes of the mouse pox virus in Australia.
- Transgenic Animals in Xenotransplantation: Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.
- Researchers are also discovering the use of cell transplantation therapy for patients with critical disorders such as Parkinson's or spinal cord injury. Genetic manipulation of stem cells which involves the growth of tissues on scaffolding can be utilized as a provisional skin alternate for healing wounds or burns. Tissue engineering is rapidly becoming an appropriate substitute in methodology that involves replacement of human structure such as cerebrospinal shunts, heart valves, cartilage and other organs.
- Transgenic combinations might help researchers to develop vaccines against deadly diseases. For instance, the DNA of human tumour fragments is inserted into tobacco plants in order to develop a vaccine against non-Hodgkin's lymphoma.

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- Likewise, researchers have produced a flu vaccine using human DNA and tobacco plants.
- Transgenic Animals in Blood Replacement: Transgenic swine are used to produce human haemoglobin.
- Transgenic animals are used in toxicity testing.
- Transgenic Animals in Agriculture: Transgenic animals are used for milk production. Generally, transgenic mice help in improving the composition of milk. Similarly, transgenic pigs are used to augment milk production by changing the composition of lactose. Further, transgenic sheep are also used for production of wool. Researchers all over the world are putting in serious efforts to generate disease-resistant animals like influenza-resistant pigs.

6.4 CREATION OF DOLLY - THE SHEEP

Dolly, born on 5th July, 1996, at the Roslyn Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell. As the Dolly's DNA came from a mammary gland cell, she was named after the country singer Dolly Parton. 'Dolly' was created using the technique of Somatic Cell Nuclear Transfer (SCNT). The details of technique are discussed below:

- Firstly, the nucleus of a somatic (body) cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed).
- Secondly, the somatic nucleus present inside the egg, is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg).
- Thirdly, zygote is stimulated to undergo division divide by an electric shock.
- Lastly, when the developing zygote forms a blastocyst, it is implanted in the womb of a surrogate mother.

6.4.1 Steps Involved in the Production of Dolly

1. Dolly had three mothers namely:
   - First mother provided the egg,
   - Second mother provided the DNA,
   - Third mother (surrogate mother) carried the cloned embryo to term.
2. Scottish Blackface ewes were treated with Gonadotropin-Releasing Hormone (GnRH) to cause them to produce oocytes ready to be fertilized.\

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3. Plunge a micropipette into the egg over the polar body to remove the polar body as well as the haploid pronuclear within the egg.
4. Fuse each enucleated egg with a diploid cell growing in culture.
5. Somatic cells from the mammary gland of an adult Finn Dorset ewe are grown in tissue culture.
6. Donor cells and enucleated recipient cells are placed together in tissue culture.
7. The cultures are then exposed to brief pulses of electricity in order to stimulate their respective plasma membranes to fuse and begin the process of mitosis, i.e., cell division.
8. Zygotes are then developed into tissue culture media until they grows into a blastocyst.
9. Numerous blastocysts were transferred into the uterus of Scottish Blackface ewes for implantation.
10. One ewe gave birth to Dolly after 148 days.

6.4.2 Probability of Dolly's Birth

- 277 diploid somatic cells were fused with 277 enucleated unfertilized eggs.
- 29 viable reconstructed eggs survived and were implanted in surrogate Blackface ewes.
- 1 gave birth to Dolly.

6.4.3 Life and Death of Dolly

- Dolly lived her entire life at the Roslyn Institute in Midlothian.
- 'Dolly' was bred with a 'Welsh Mountain Ram'.
- She produced six lambs.
- Bonnie (born in April 1998) was her first lamb.
- Subsequently, she produced twin lambs named Sally and Rosie.
- In the year 2000, she gave birth to triplets- Lucy, Darcy and Cotton.
- On 14 February 2003, Dolly was euthanized as she had developed a progressive lung cancer called ovine pulmonary adenocarcinoma as well as severe arthritis.
- Dolly lived for 6.5 years.

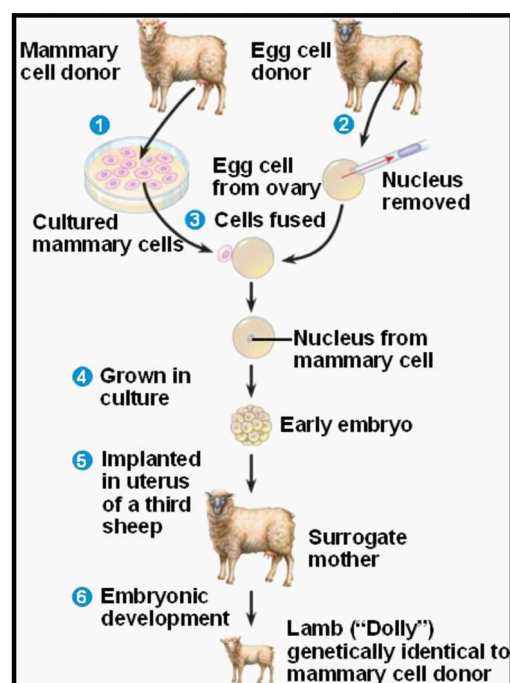


Fig. 6.6 *Steps Involved in the Cloning Process of Dolly- the Sheep*

Check Your Progress

1. Explain the process of DNA microinjection.
2. Define embryonic stem cells.
3. Elaborate on the transgenics.
4. What do you understand by the xenotransplantation?
5. Illustrate the retrovirus-mediated gene transfer.

6.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. DNA microinjection involves the direct microinjection of the desirable gene construct (either a single gene or a grouping of desirable genes) from another individual of the same species or from an entirely different species, into the pronuclear of a fertilized ovum.
2. Embryonic Stem Cells (ESCs) are derived from early stages of embryo development, i.e., from mouse or human blastocyst. They are pluripotent, self-renewing cells .They can be stored in culture for a long duration of time as undifferentiated cell lines. These undifferentiated cells can be stimulated to differentiate into any cell line ESCs can differentiate into endoderm, mesoderm, and ectoderm embryonic germ layers or any type of somatic cells. They are of wide significance in tissue regeneration therapy.

3. Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding.
4. Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.
5. Retrovirus-mediated gene transfer is mediated by means of a carrier or vector, normally a virus or a plasmid. Retroviruses are the most common choice to be used as vectors to transfer genetic material (DNA or RNA) into the cell, taking benefit of their capability to infect host cells in this way. Offspring resulting from this technique are chimeric animals, i.e., not all of them carrying the retrovirus. Transmission of the foreign gene is achievable only if the retrovirus incorporates into some of the germ cells.

6.6 SUMMARY

- The foundation for the production of transgenic animals began using sperm mediated gene transfer and in 1980s, the transgenic mice are produced via using the most accepted microinjection technique. Subsequently, production of several transgenic animals was reported in 1985.
- There are several types of transgenic animals like transgenic chickens, sheep, pigs, birds, and insects, etc. Microinjection was one of the widely accepted and frequently methodology used for the process of transgenics, however, it has some major drawbacks like low competence, and inconsistent expression patterns, etc.
- Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species.
- A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding.
- The basic principle in the production of transgenic animals is the introduction of a foreign gene or genes into an animal. The new inserted foreign gene is referred to as the transgene.
- Stem cells are undifferentiated cells that have the potency to differentiate into different type of cell (somatic and germ cells) and therefore to give rise to a complete organism.

- Retrovirus-mediated gene transfer is mediated by means of a carrier or vector, normally a virus or a plasmid. Retroviruses are the most common choice to be used as vectors to transfer genetic material (DNA or RNA) into the cell, taking benefit of their capability to infect host cells in this way.
- Transgenic Animals are widely used as Disease Model: Historically, mice have been used to model human disease because of their physiological, anatomical and genomic similarities to humans.
- Transgenic animals such as mice are quite popular with researchers to study critical diseases such as Alzheimer's, cancer, and AIDS. Transgenic animals enable scientists to be aware of the role of genes in specific diseases.
- Dolly, born on 5th July, 1996, at the Roslyn Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell. As the Dolly's DNA came from a mammary gland cell, she was named after the country singer Dolly Parton.
- On 14 February 2003, Dolly was euthanized as she had developed a progressive lung cancer called ovine pulmonary adenocarcinoma as well as severe arthritis.

6.7 KEY WORDS

- **Production of transgenic animals:** The foundation for the production of transgenic animals began using sperm mediated gene transfer and in 1980s, the transgenic mice are produced via using the most accepted microinjection technique.
- **Types of transgenic animals:** There are several types of transgenic animals like transgenic chickens, sheep, pigs, birds, and insects, etc.
- **Transgenics:** Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species.
- **Retrovirus-mediated gene transfer:** Retrovirus-mediated gene transfer is mediated by means of a carrier or vector, normally a virus or a plasmid.
- **Dolly:** Dolly, born on 5th July, 1996, at the Roslyn Institute in Edinburgh, Scotland, was a female Finn Dorset sheep. It was the first mammal cloned from an adult somatic cell.

6.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. State the process of DNA microinjection.

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2. Explain the embryonic stem cells.
3. Interpret the transgenics.
4. Elaborate on the xenotransplantation.
5. Define the retrovirus-mediated gene transfer.

Long-Answer Questions

1. Discuss the methods of producing transgenic animals.
2. Write down the applications of using transgenic animals.
3. Elaborate the process of production of 'Dolly- the Sheep'.
4. Explain the xenotransplantation and its applications with the help of examples.
5. Analyse the entire process of creating transgenic animals.

6.9 FURTHER READINGS

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UNIT 7 TRANSGENIC ANIMALS FROM FOETAL CELLS

NOTES

Structure

7.0 INTRODUCTION

Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding. The foundation for the production of transgenic animals began using sperm mediated gene transfer and in 1980s, the transgenic mice are produced via using the most accepted microinjection technique. Subsequently, production of several transgenic animals was reported in 1985.

There are several types of transgenic animals like transgenic chickens, sheep, pigs, birds, insects etc. Microinjection was one of the widely accepted and frequently methodology used for the process of transgenic, however, it has some major drawbacks like low competence, inconsistent expression patterns etc. Hence, researchers began to look for alternative methods like intracytoplasmic injection of sperm heads carrying foreign DNA, sperm-mediated DNA transfer, injection or infection of oocytes or via different types of viral vectors, RiboNucleic Acid (RNA) interference technology as well as the use of nuclear transfer technology. Though, there are several social, extrinsic, and intrinsic ethical issues as well as legal implications are associated with the transgenic animals.

However, transgenic animals have several potential use like Transgenic animals are widely used as disease model, Transgenic animals as food, Transgenic animals in Drug and Industrial production, Transgenic animals in Disease control, Transgenic animals in Xenotransplantation, Transgenic combinations might help researchers to develop vaccines against deadly diseases, Transgenic animals in Blood replacement, Transgenic animals are used in toxicity testing, Transgenic animals in Agriculture.

In this unit, you will study about the transgenic animals from foetal cells, transgenic animals in xenotransplantation, transgenic organisms to interrupt disease cycles, artificial insemination, and embryo transfer technology.

7.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the concept transgenic animals from foetal cells
- Define the transgenic animals in xenotransplantation
- Analyse the transgenic organisms to interrupt disease cycles
- Elaborate on the artificial insemination
- Comprehend the embryo transfer technology

7.2 TRANSGENIC ANIMALS FROM FOETAL CELLS

Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.

Stem cells have unique properties:

1. They are unspecialized cells
2. They can undergo self-replication via asymmetric cell division.
3. They can undergo differentiation to produce specific cells in accordance with the need of the body as well as signal received by the stem cells
4. Fetal stem cells have characteristic features:
   - These cells are present in the organs of the foetuses.
   - They are capable of differentiating into two different types of stem cells, i.e., pluripotent stem cells and hematopoietic stem cells.

Fetal cells like fibroblasts are totipotent. Fetal cell cloning was fruitfully carried out by some researchers to create transgenic sheep (Dolly), transgenic bull calf (Gene) as well as other transgenic animals. In this methodology, transgenes (foreign genes) can be inserted into the fetal cell genomes to produce desirable products by transgenic animals. The steps involved in the technique are discussed below:

1. Foetal cell like Fibroblasts were collected from a fifty five day old bovine fetus.

2. Foetal fibroblasts cells exhibit totipotency and are grown further in a nutritious culture medium.
3. Transgene having the required desirable character can be introduced into fibroblasts via microinjection or any other suitable molecular technique.
4. The Fibroblast cell nucleus having the genetically altered DNA is removed from the cell.
5. It is then inserted into a bovine enucleated ovum (enucleated ovum refers to the ovum lacking nucleus).
6. This ovum cell undergoes division to form blastocyst in a culture medium which is then implanted in a surrogate (foster) mother cow to produce transgenic calves.

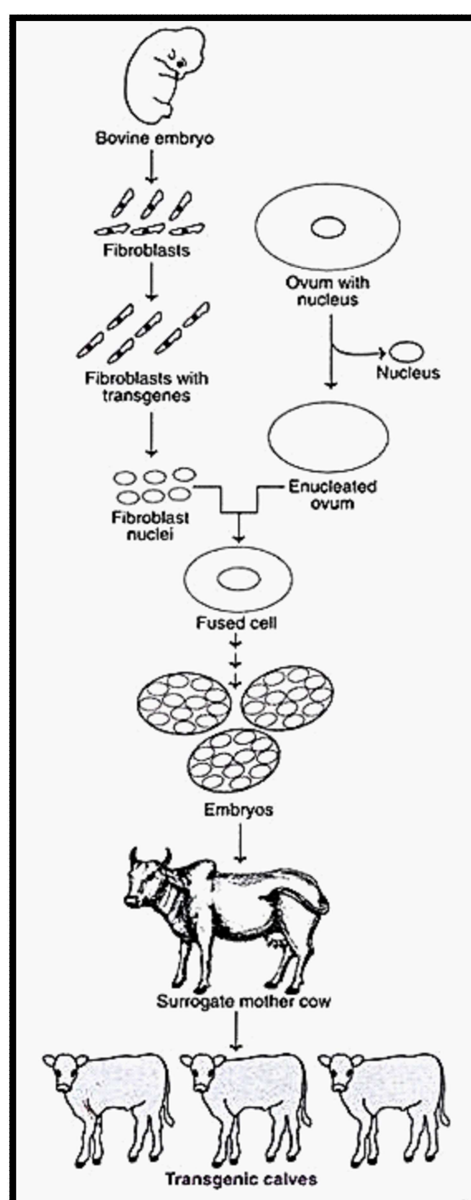


Fig. 7.1 *Production of Transgenic Calves by Nuclear Transfer Technology*

7.3 TRANSGENIC ANIMALS IN XENOTRANSPLANTATION

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Transplantation of organs such as liver, kidney, and heart, etc.in humans has developed into one of the most sophisticated surgical practice to replace the non-functional or defective organs with the functional one. However, as the demand for human organs rises, the shortage of organ donors becomes one of the major limiting factors. This has led to delay and unnecessary death of patients in need of precious organs. Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients. However, organ rejection occurs frequently due to following reasons:

A. Antibodies raised by the recipient against the transplanted foreign organ.

B. Activation of host's complement system due to transplantation.

Researchers are conducting research actively to utilize pig's organs as a substitute for replacing defective human organs. They have recognized that the primary reason for rejection of pig's organs by primates is due to the existence of an exceptional cluster of disaccharides (Gal-á 1, 3-Gal) synthesized by the enzyme á 1, 3-galactosyltransferase, present in pigs but absent in primates. Scientists are hopeful that knockout methodology i.e. by knocking the gene encoding the enzyme á 1, 3-galactosyltransferase in pigs can be developed in the next few years. Another approach can be introduction of the gene encoding the enzyme á 1, 3-galactosyltransferase in primates. Researchers are optimistic that successful implementation of the procedure will reduce immune reaction in the recipient.

By the above knock-out approach, researchers are likely to overcome immediate hyperactive rejection of the foreign organs. Subsequently, the next problem is the delayed rejection of the foreign organ which involves cells like macrophages and natural killer cells of the host. Additional, concern of xenotransplantation is that the endogenous pig retroviruses might get triggered after organ transplantation which can result in novel genetic alterations with unidentified consequences.

Even though, the ideas looks great as proposal yet the utilization of transgenic animals in xenotransplantation is still at the experimental stages and it is highly doubtful whether this will become a reality in the near future.

Further, concept of xenotransplantation is associated with ethical issues and hence bioethicists as well as the general public are against it.

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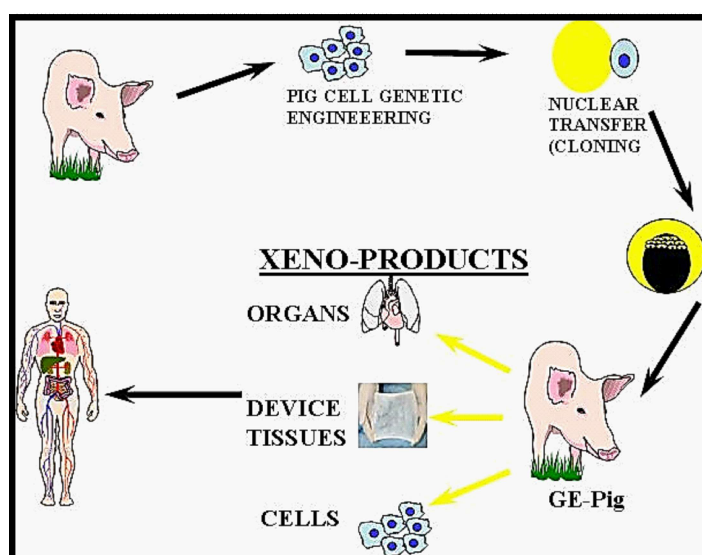


Fig. 7.2 *Use of Transgenic Pig in the Process of Xenotransplantation*

7.4 TRANSGENIC ORGANISMS TO INTERRUPT DISEASE CYCLES

Trans-genesis will be useful in the control of human diseases by interrupting the life cycle of the parasite. Several attempts have been made in the recent years in this field; however the success rate is very low. In the future, transgenic organisms might provide as environmental replacements to manage numerous diseases. In the next section we will be discussing a few such animals:-

7.4.1 Transgenic Snails

Schistosomiasis is a parasitic diseases caused by Schistosoma and characterized by symptoms such as fever, diarrhoea, chills, fever, intestinal ulceration, etc. The parasite transmits disease to humans by penetrating via skin while contacted in water. By means of developing transgenic snails, it is quite possible to interrupt the life cycle of Schistosoma. A few researchers have tried to produce transgenic snails that will prevent the invasion of the parasite. In the future, it will become possible to break the life cycle of Schistosoma If such snails are released into the environment.

7.4.2 Transgenic Mosquitoes

Female Anopheles mosquitoes are responsible for the transmission of Plasmodium species causing a deadly disease known as malaria. Researchers have identified certain critical genes responsible for the transmission of the parasite. In theory, it is quite possible to modify these critical genes in order to produce transgenic mosquito. Release of transgenic mosquitoes into the environment in large numbers, will somehow dilute the population of native female Anopheles mosquitoes, and thus can prevent or reduce the transmission of malaria causing parasite.

7.4.3 Transgenic Bollworms

Bollworm is the common term used for the moth larvae responsible for damaging a variety of crops. Researchers have developed transgenic bollworms with a foreign 'Suicide Gene'. When released into the environment in large numbers, the transgenic bollworms will mate with wild bollworms and produce larvae that will die due to the expression of the suicidal gene. A large number of variety of crops can be saved by adopting this strategy.

7.4.4 Transgenic Medflies

Mediterranean fruit flies are responsible for destroying fruit as well as coffee crops throughout the world. Efforts are being done to replace wild medflies with transgenic medflies in order to save the crops for human consumption.

7.4.5 Transgenic Tsetse Flies

African sleeping sickness- a protozoal disease is transmitted by tsetse fly. This disease is responsible for affecting the nervous system which subsequently leads to coma. Researchers working on the tsetse flies have identified a protein capable of killing disease-causing protozoa. They created the transgenic tsetse fly by inserting the foreign gene encoding for the killer protein in the gut bacteria of the tsetse fly. In this way, it is possible to prevent the transmission of disease.

7.5 ARTIFICIAL INSEMINATION

Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female. There are three common methods of Artificial Insemination (AI):

A. Vaginal method

B. Recto vaginal method

C. The speculum method.

Based on the size as well as the structure of the bovine reproductive tract, two methods namely Recto vaginal method and the speculum method are widely accepted as suitable methods for insemination in beef cattle. Below, we will discuss both the methods in detail:

1. Recto Vaginal Method: This is one of the most widely accepted methodology in dairy industry due to its practicality as well as it is believed to be the safest for the animal. This methodology involves the manipulation of the reproductive tract, primarily the cervix, through rectal palpation.
2. The Speculum Method: This methodology involves placing a speculum (or spectrum) into the vagina pushed up to the posterior end of the cervix.

7.5.1 Benefits of Artificial Insemination

- Increase in efficiency of usage of genetically superior bull.
- Increased potential for genetic selection.
- Cheap and economical over a long period of time.
- Protecting the interest of both dairy farmers as well as animals.
- Reduction in the transmission of diseases.

7.5.2 Drawbacks of Artificial Insemination

- More laborious technology requires skilled labour.
- Dairy farmer needs to detect the most genetically fit male to carry out the process of Artificial insemination.
- It can decrease the genetic variability over a long period of time.

7.6 EMBRYO TRANSFER TECHNOLOGY (ETT)

The first Embryo Transfer Technology (ETT) project in the country was initiated by NDDB (National Dairy Development Board) in 1987 by the establishment of a central ET laboratory at Sabarmati Ashram Gaushala (SAG), Bidaj. The project was funded by the Department of Biotechnology (DBT), Ministry of Science & Technology, GoI for 5 years i.e. from April 1987 to March 1992. Under this project, NDDB established one Main ET Lab at SAG Bidaj and four Regional ET Labs at CFSP&TI, Hessarghatta (Karnataka), ABC, Salon (UP), Shri Nashik Panchavati Panjrapole, Nashik (Maharashtra) and Buffalo Breeding Centre, Nekarikallu (AP). NDDB also assisted in establishment of 14 State ET centres across the country.

'Embryo Transfer' (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals. Usually, one can get one calf from a superior quality female dairy animal in a year. However, by using 'MOET /ET' technology, dairy workers can get 10-20 calves in a year from a cow/buffalo. A genetically superior cow/buffalo is administered artificial hormones with FSH-like activity to induce super-ovulation. Under the influence of the FSH-like hormone, the genetically superior female produces numerous eggs instead of single egg produced in general. The super-ovulated female is artificially inseminated multiple time (2-3 time) at 12 hour interval during oestrus and on 7th day post insemination the uterus of the artificially inseminated female is flushed with a medium to recover the developing embryos. Embryos are collected along with flushing medium in a specific filter. Thereafter, the quality of the developing embryos is assessed under the microscope. Superior quality embryos are either preserved or frozen for the purpose of transfer in future or they are transferred into recipient females (surrogate

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mother) roughly seven days post the heat date. Thus, with the use of 'ET' technology numerous genetically superior calves can be produced in a year.

SAG has done ground-breaking work in this field and so far has produced 14,388 viable embryos and 755 calves, which is maximum by any organisation in the country. Of these, 1026 embryos are of indigenous cattle breeds, from which 122 calves have been born. Besides these, around 3000 embryos of buffalo breeds have also been produced. Under the project, the first buffalo calf of India from frozen thawed embryo was born in the year 1991.

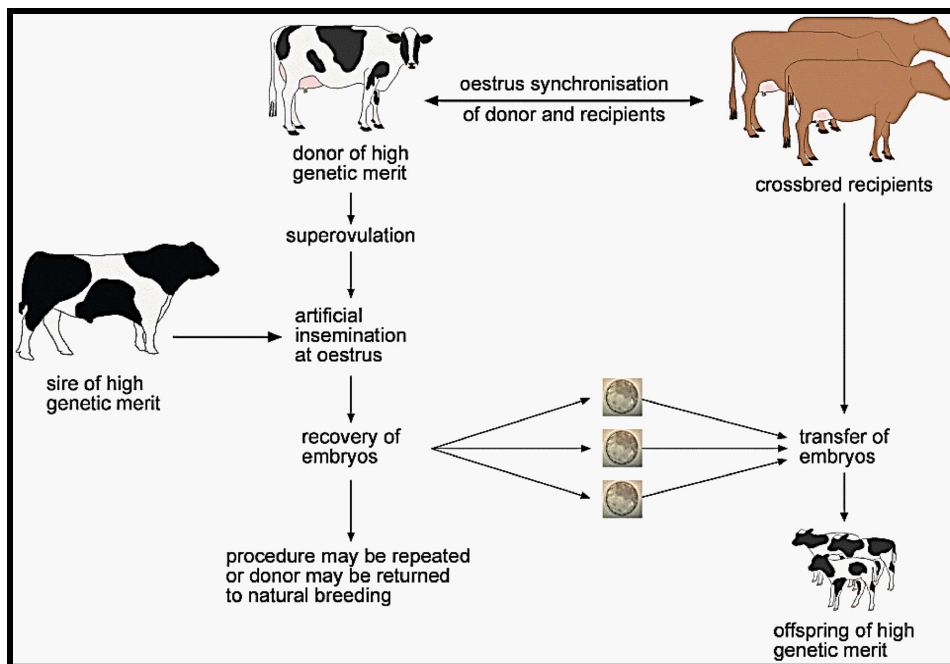


Fig. 7.3 *Embryo Transfer Technology*

Check Your Progress

1. Explain the term xenotransplantation.
2. Define artificial insemination.
3. Elaborate on the foetal stem cells.
4. What is 'ET'-Embryo transfer technology used for?
5. Differentiate between recto vaginal method and speculum method.

7.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of

precious human organs like hearts and kidneys. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.

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2. Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female.
3. Fetal stem cells have characteristic features:-These cells are present in the organs of the fetuses and they are capable of differentiating into two different types of stem cells, i.e., pluripotent stem cells and hematopoietic stem cells. Fetal cells like fibroblasts are totipotent. Fetal cell cloning was fruitfully carried out by some researchers to create transgenic sheep (Dolly), transgenic bull calf (Gene) as well as other transgenic animals.
4. Embryo Transfer (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals.
5. Recto Vaginal method is one of the most widely accepted methodology in dairy industry due to its practicality as well as it is believed to be the safest for the animal. This methodology involves the manipulation of the reproductive tract, primarily the cervix, through rectal palpation whereas the speculum method involves placing a speculum (or spectrum) into the vagina pushed up to the posterior end of the cervix.

7.8 SUMMARY

- Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
- Fetal cells like fibroblasts are totipotent. Fetal cell cloning was fruitfully carried out by some researchers to create transgenic sheep (Dolly), transgenic bull calf (Gene) as well as other transgenic animals.
- Transplantation of organs such as liver, kidney, heart, etc.in humans has developed into one of the most sophisticated surgical practice to replace the non-functional or defective organs with the functional one.
- By the above knock-out approach, researchers are likely to overcome immediate hyperactive rejection of the foreign organs. Subsequently, the next problem is the delayed rejection of the foreign organ which involves cells like macrophages and natural killer cells of the host.
- Trans-genesis will be useful in the control of human diseases by interrupting the life cycle of the parasite. Several attempts have been made in the recent years in this field; however the success rate is very low.
- Schistosomiasis is a parasitic diseases caused by Schistosoma and characterized by symptoms such as fever, diarrhoea, chills, fever, and intestinal

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ulceration, etc. The parasite transmits disease to humans by penetrating via skin while contacted in water.

- Female Anopheles mosquitoes are responsible for the transmission of Plasmodium species causing a deadly disease known as malaria. Researchers have identified certain critical genes responsible for the transmission of the parasite.
- Bollworm is the common term used for the moth larvae responsible for damaging a variety of crops. Researchers have developed transgenic bollworms with a foreign 'Suicide Gene'.
- Mediterranean fruit flies are responsible for destroying fruit as well as coffee crops throughout the world. Efforts are being done to replace wild medflies with transgenic medflies in order to save the crops for human consumption.
- African sleeping sickness- a protozoal disease is transmitted by tsetse fly. This disease is responsible for affecting the nervous system which subsequently leads to coma.
- Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female.
- Embryo Transfer (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals.
- Under the influence of the FSH-like hormone, the genetically superior female produces numerous eggs instead of single egg produced in general.

7.9 KEY WORDS

- **Stem cells:** Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
- **Fetal cells:** Fetal cells like fibroblasts are totipotent. Fetal cell cloning was fruitfully carried out by some researchers to create transgenic sheep (Dolly), transgenic bull calf (Gene) as well as other transgenic animals.
- **Xenotransplantation:** Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys.
- **Transgenesis:** Trans-genesis will be useful in the control of human diseases by interrupting the life cycle of the parasite. Several attempts have been made in the recent years in this field; however the success rate is very low.
- **Schistosomiasis:** Schistosomiasis is a parasitic diseases caused by Schistosoma and characterized by symptoms such as fever, diarrhoea, chills, fever, and intestinal ulceration, etc.

- **Transgenic bollworms:** Bollworm is the common term used for the moth larvae responsible for damaging a variety of crops. Researchers have developed transgenic bollworms with a foreign 'Suicide Gene'.
- **Artificial insemination:** Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female.

7.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Define the term xenotransplantation.
2. State artificial insemination.
3. Illustrate the foetal stem cells.
4. What is embryo transfer technology used for?
5. Differentiate between recto vaginal method and speculum method.

Long-Answer Questions

1. Briefly discuss the process of artificial insemination.
2. Describe the embryo transfer (ET) technology with the help of example.
3. Analyse the role of transgenic organisms to interrupt disease cycles.
4. Explain the process of nuclear transfer technology. Give appropriate examples.
5. Define the advantages and drawbacks of the process of artificial Insemination.

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BLOCK - III
MOLECULAR DIAGNOSTIC TECHNIQUES AND PHEROMONES

UNIT 8 ISOLATION AND PURIFICATION OF NUCLEIC ACIDS

Structure

8.0 INTRODUCTION

The first isolation of nucleic acid was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits used for extraction, and selecting the correct one will save time on kit optimization and extraction procedures. PCR sensitivity detection is considered to show the variation between the commercial kits.

Some of the most common DNA extraction methods include organic extraction, Chelex extraction, and solid phase extraction. These methods consistently yield isolated DNA, but they differ in both the quality and the quantity of DNA yielded. When selecting a DNA extraction method, there are multiple factors to consider, including cost, time, safety, and risk of contamination.

Every gene manipulation procedure requires genetic materials like DNA and RNA. Nucleic acids occur naturally in association with proteins and lipoprotein organelles. The dissociation of a nucleoprotein into nucleic acid and protein moieties and their subsequent separation, are the essential steps in the isolation of all species of nucleic acids.

Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.

Isolating the genetic material (DNA) from cells (Bacterial, Viral, Plant or Animal) involves three basic steps: (i) Rupturing of cell membrane to release the cellular components and DNA, (ii) Separation of the nucleic acids from other cellular components, and (iii) Purification of nucleic acids.

In this unit, you will study about the isolation and purification of nucleic acids, and hybridization: Southern, Western, and Northern hybridization.

8.1 OBJECTIVES

After going through this unit, you will be able to:

- Explain the isolation and purification of nucleic acids
- Define the hybridization: Southern, Western, and Northern hybridization

8.2 ISOLATION AND PURIFICATION OF NUCLEIC ACIDS

Every gene manipulation procedure requires genetic materials like DNA and RNA. Nucleic Acids occur naturally in association with Proteins and Lipoprotein Organelles. The dissociation of a nucleoprotein into Nucleic Acid and Protein moieties and their subsequent separation, are the essential steps in the isolation of all species of Nucleic Acids. Isolation of Nucleic Acids is followed by quantitation of Nucleic Acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.

Isolating the genetic material (DNA) from cells (Bacterial, Viral, Plant or Animal) involves three basic steps:

- Rupturing of cell membrane to release the cellular components and DNA.
- Separation of the Nucleic Acids from other cellular components.
- Purification of Nucleic Acids.

I. Isolation and Purification of Genomic DNA

Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell (Refer Figure 8.1).

The method of isolation of genomic DNA from a bacterium comprises following steps:

- Bacterial culture growth and harvest.
- Cell wall rupture and cell extract preparation.
- DNA purification from the cell extract.
- Concentration of DNA solution.

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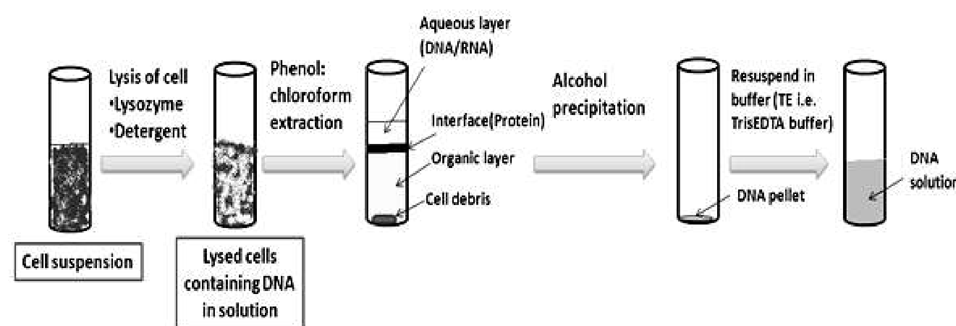


Fig. 8.1 *Isolation of Genomic DNA*

Growth and Harvest of Bacterial Culture: Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less of the initial culture volume.

Preparation of Cell Extract: Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of *Escherichia coli* comprising multilayered cell wall. The lysis of cell wall to release the genetic material, i.e., DNA can be achieved by following ways:

- Physical method by mechanical forces.
- Chemical method by metal chelating agents, i.e., EDTA and surfactant, i.e., SDS or enzyme (for example, lysozyme).

  The lysozymes are present in egg-white, salivary secretion and tears, and catalyzes the breakdown of cell wall, i.e., the peptidoglycan layer. The EDTA (Ethylene Diamine Tetra-Acetic Acid) is a chelating agent necessary for destabilizing the integrity of cell wall, and inhibits the cellular enzymes that degrade DNA. The SDS (Sodium Dodecyl Sulphate) helps in removal of lipid molecules and denaturation of membrane Proteins. Generally, a mixture of EDTA and lysozyme is used. Cell lysis is followed by centrifugation to pellet down the cell wall fractions leaving a clear supernatant containing cell extract.

- **Purification of DNA:** In addition to DNA, a cell extract contains significant quantities of Protein and RNA which can be further purified by following methods:
- **Organic Extraction and Enzymatic Digestion for Removal of Contaminants:** It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for Protein separation. The Proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with Pronase or Protease, in addition to Phenol/ Chloroform, ensures complete removal

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of Proteins from the extract. The RNA can be effectively removed by using ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

- **Using Ion-Exchange Chromatography:** This involves the separation of ions and polar molecules (Proteins, Small nucleotides and Amino Acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.
- **Concentration of DNA Samples:** Concentration of DNA can be done using ethanol along with salts, such as Sodium acetate, Potassium acetate, etc. These salts provide metal ions like Sodium ions (Na^+), Potassium ions (K^+) which help in aggregation and hence precipitation of DNA molecules.

II. Isolation and Purification of Plasmid DNA

This comprises growth of the bacterial cell, harvesting and lysis of the bacteria, and purification of the plasmid DNA. The growth of the bacterial cell involves growth of the bacterial cells in a media containing essential nutrients. The harvest and lysis of bacteria results in the precipitation of DNA and cellular Proteins. Addition of acetate-containing neutralization buffer results in the precipitation of large and less supercoiled chromosomal DNA and Proteins leaving the small bacterial DNA plasmids in solution. The purification is same for both plasmid and genomic but former involves an additional step, i.e., the separation of plasmid DNA from the large bacterial chromosomal DNA.

III. Methods for Separation of Plasmid DNA

Separation of plasmid DNA is based on the several features like size and conformation of plasmid DNA and bacterial DNA. Plasmids are much smaller than the bacterial main chromosomes, the largest plasmids being only 8% of the size of the *Escherichia coli* chromosome. The separation of small molecules (i.e., plasmids) from larger ones (i.e., bacterial chromosome) is based on the fact that plasmids and the bacterial chromosomes are circular but bacterial chromosomes break into linear fragments during the preparation of the cell extract resulting in separation of pure plasmids. The methods of separation of plasmid DNA are described as below (Refer Figure 8.2):

Separation Based on Size Difference

- It involves lysis of cells with lysozyme and in the presence of sucrose (prevents the immediate bursting of cell).
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmic membrane called as sphaeroplasts.

- Cell lysis is then induced by the addition of a non-ionic detergent (for example, Triton X-100) or ionic detergents (for example, SDS) causing chromosomal breakage.
- Bacterial chromosome attached to cell membrane, upon lysis gets removed with the cell debris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very little breakage of the bacterial DNA.

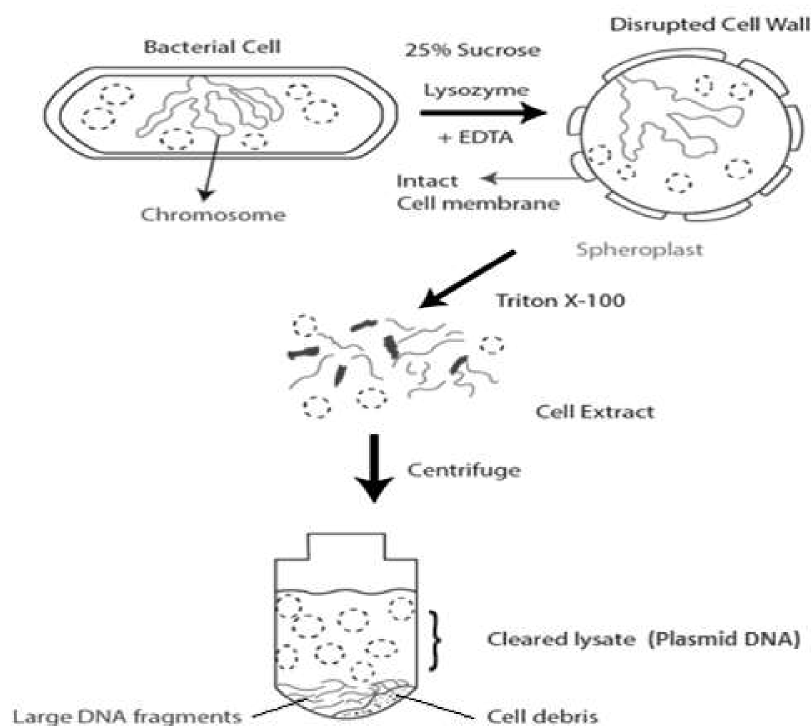


Fig. 8.2 *Separation of Plasmid DNA on the Basis of Size*

Separation Based on Conformation: Plasmids are supercoiled molecules formed by partial unwinding of double helix of the plasmid DNA during the plasmid replication process by enzymes called topoisomerases. The supercoiled conformation can be maintained when both polynucleotide strands are intact, hence called Covalently Closed-Circular (CCC) DNA. If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state taking an alternative conformation, called Open-Circular (OC). Super coiling is important in plasmid preparation due to the easy separation of supercoiled molecules from non-supercoiled ones (Refer Figure 8.3).

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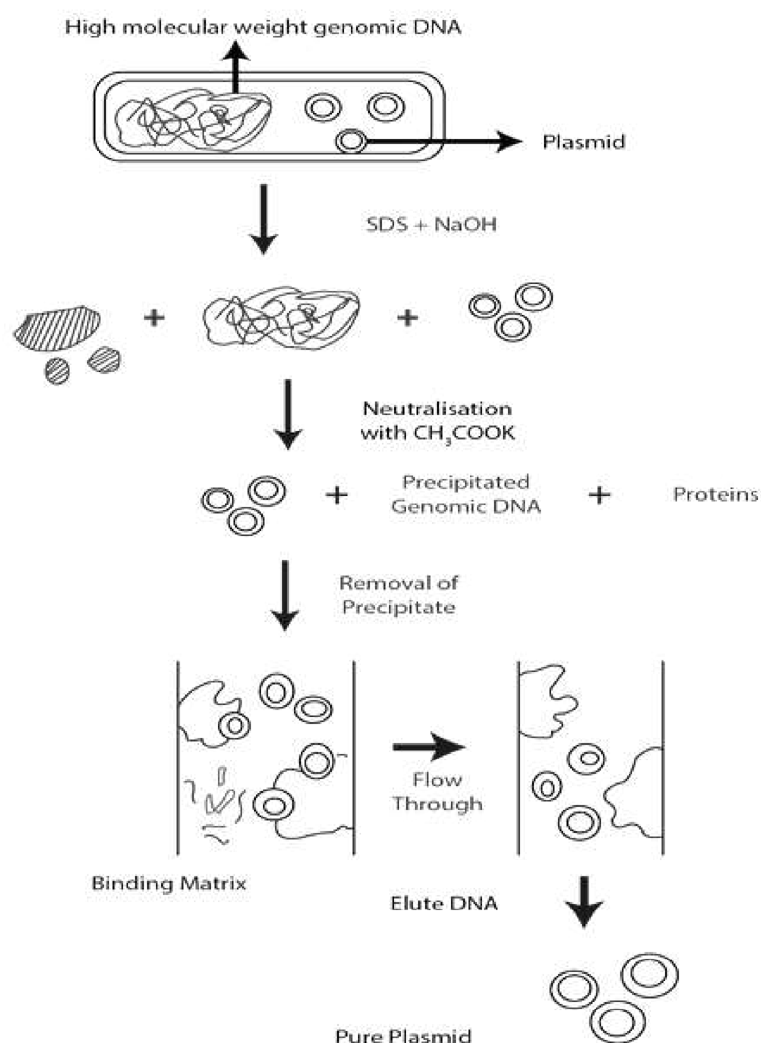


Fig. 8.3 Separation of Plasmid DNA Based on Conformation by Alkaline Denaturation Method

8.3 HYBRIDIZATION: SOUTHERN, WESTERN, AND NORTHERN

Blotting techniques were developed to discriminate fragments of nucleic acids. These techniques involve several processes; electrophoresis is one of the processes and is used to separate fragments of DNA and RNA. In Southern blotting, named after Edward Southern, restriction enzymes cut fragments of DNA are separated by AGE (Agarose Gel Electrophoresis) or PAGE (PolyAcrylamide Gel Electrophoresis), transferred to a membrane or blot, and visualized by hybridization with labeled probes. Northern blotting, not named after an inventor but by analogy to Southern blotting, separates RNA. RNA molecules are shorter and have defined lengths, cutting by restriction enzymes is not required. Denaturing conditions are

required because of RNA secondary structures. After membrane blotting, the separated types of RNA are visualized with staining or labeled probes. Western blotting, not named after an inventor but by analogy to Southern blotting, does not separate nucleic acids, it separates Proteins in a mixture. The Proteins are usually separated with PAGE, transferred to the membrane and visualized with a labeled antibody against the Proteins of interest. Following gel electrophoresis, probes are often used to detect specific molecules from the mixture. However, probes cannot be applied directly to the gel. The problem can be solved by three types of blotting methods: Southern blotting, Northern blotting and Western blotting. Southern blotting is a technique for detecting specific DNA fragments in a complex mixture. The technique was invented in mid-1970s by Edward Southern. It has been applied to detect Restriction Fragment Length Polymorphism (RFLP) and Variable Number of Tandem Repeat (VNTR) Polymorphism. The latter is the basis of DNA fingerprinting. Polymorphism refers to the DNA sequence variation between individuals of a species. If the sequence variation occurs at the restriction sites, it could result in RFLP. The most well-known example is the RFLP due to β Globin Gene Mutation. VNTR may result from unequal crossover. It is the molecular basis of DNA fingerprinting which has many practical applications.

Northern blotting is used for detecting RNA fragments, instead of DNA fragments. The technique is called 'Northern' simply because it is similar to 'Southern', not because it was invented by a person named 'Northern'. In the Southern blotting, DNA fragments are denatured with alkaline solution. In the Northern blotting, RNA fragments are treated with formaldehyde to ensure linear conformation. Western blotting is used to detect a particular Protein in a mixture. The probe used is therefore not DNA or RNA, but antibodies. The technique is also called 'Immune Blotting'.

Dot and Slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a Nitrocellulose or Nylon Membrane. Hybridization analysis can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations. Dot and slot blots differ only in the geometry of the blot, a series of spots giving a hybridization pattern that is amenable to analysis by densitometric scanning. Samples are usually applied to the membrane using a manifold attached to a suction device. The basic protocol describes such a procedure for dot or slot blotting on an uncharged nylon membrane, annotations to the steps detail the minor modifications that are needed if blotting onto Nitrocellulose. The first alternate protocol describes the more major changes required for blotting with a positively charged nylon membrane. A second alternate protocol describes preparation of dot blots by spotting the samples onto the membrane by hand.

Blotting Techniques and Biochemistry

Biochemistry studies the molecules, such as DNA, RNA and Proteins while the Blotting techniques are the methods that scientists use to separate these types of

molecules. In cells, they exist as a mixture. Blotting is generally done by letting a mixture of DNA, RNA or Protein flow through a slab of gel. This gel allows small molecules to move faster than bigger ones. The separated molecules are then pressed against a membrane, which helps move the molecules from the gel onto the membrane. The molecules stick to the membrane, but stay in the same location, apart from each other, as if they were still in the gel.

Consequently blots are techniques for transferring DNA, RNA and Proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis. The Southern blot is used for transferring DNA, the Northern blot for RNA and the Western blot for Protein. South-Western blotting is used to investigate DNA–Protein interactions. The advantage of this technique over other related methods, such as Electrophoretic Mobility Shift Assay (EMSA) and DNA foot printing is that it provides information regarding the molecular weight of unknown Protein Factor. This method combines the features of Southern and Western blotting techniques, a denaturing SDS-PAGE is first employed to separate Proteins electrophoretically based on size. After transferring the Proteins to a membrane support, the membrane-bound Proteins are renatured and incubated with a ^{32}P-labeled double-stranded Oligonucleotide probe of specific DNA sequence. The interaction of the probe with the Protein(s) is later visualized by autoradiography. This technique could be combined with database searching, prediction of potential Protein factors binding onto a target motif, for example Patch search, in-vitro super shift EMSA and in-vivo Chromatin Immuno-Precipitation (ChIP) assays for effective identification of Protein factors. The whole South-Western blotting procedure takes ~ 4 days to complete. Figure 8.4 illustrates the blotting compass displaying various blotting techniques and its applications.

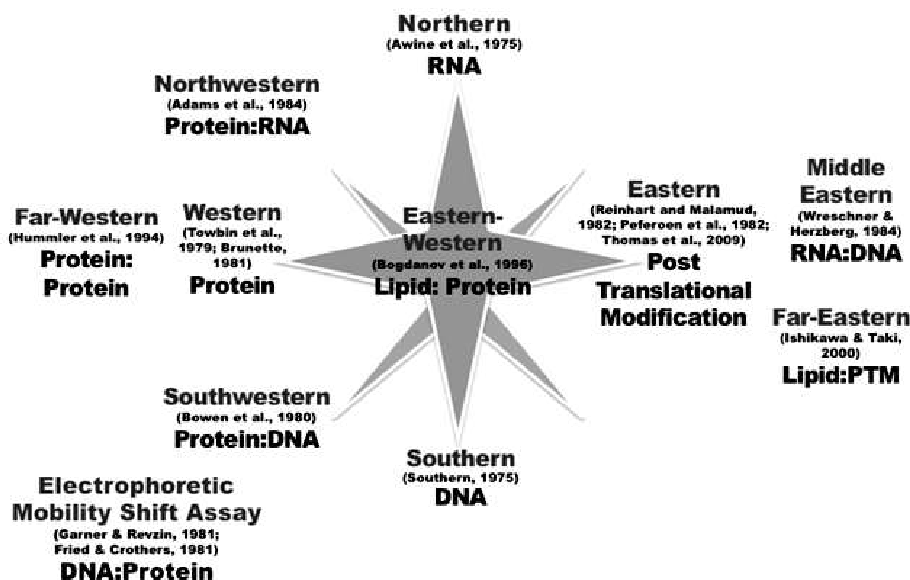


Fig. 8.4 Blotting Compass Displaying Various Blotting Techniques and its Applications

A blot, in molecular biology and genetics, is a method of transferring Proteins, DNA or RNA, onto a carrier, for example, a Nitrocellulose, PolyVinyliDene Fluoride (PVDF) or Nylon Membrane. In many examples, this is done after a gel electrophoresis, transferring the molecules from the gel onto the blotting membrane, and other times adding the samples directly onto the membrane. After the blotting, the transferred Proteins, DNA or RNA are then visualized by colorant staining, for example silver staining of Proteins, autoradiography visualization of radioactive labeled molecules performed before the blot, or specific labeling of some Proteins or Nucleic Acids. The latter is done with antibodies or hybridization probes that bind only to some molecules of the blot and have an enzyme joined to them. After proper washing, this enzymatic activity and subsequently the molecules in the blot is visualized by incubation with proper reactive, rendering either a colored deposit on the blot or a chemiluminiscent reaction which is registered by photographic film. The three main blotting techniques, namely the Western, Northern and Southern have been modified in different ways to detect slightly different molecules. The Western blot vs. the Southern blot, for example detects Protein and DNA, respectively. Each modified technique is generally done the usual way, but uses a different method to detect the molecule that is being spread out into the parallel lanes. South-Western blots detect molecules of Protein stuck to DNA, the North-Western blots detect molecules of Protein stuck to RNA and the Far-Western blots detect molecules of Protein stuck to other Proteins.

Southern Blotting

Southern blot hybridization detects target DNA fragments that have been size-fractionated by gel electrophoresis. This technique was invented in 1975 by E.M. Southern. In this technique we exploit the property of a radio-labeled probe with the single stranded DNA. If we want to detect the presence of a specific sequence in our mixed DNA sample then we will accordingly design the probe which will have complementary sequence to our target sequence.

In this procedure, called a Southern blot, DNA from the sample is cleaved into restriction fragments with a restriction endonuclease, and the fragments are spread apart by gel electrophoresis. The double-stranded helix of each DNA fragment is then denatured into single strands by making the pH of gel basic, and the gel is **'Blotted'** with a sheet of Nitrocellulose, transferring some of the DNA strands to the sheet. Next, a probe consisting of purified, single-stranded DNA corresponding to a specific gene or mRNA transcribed from that gene is poured over the sheet. Any fragment that has a nucleotide sequence complementary to the probe's sequence will hybridize (base pair) with the probe. If the probe has been labeled with ^{32}P, it will be radioactive, and the sheet will show a band of radioactivity where the probe is hybridized with the complementary fragment.

Figure 8.5 illustrates the methodology of Southern blotting technique.

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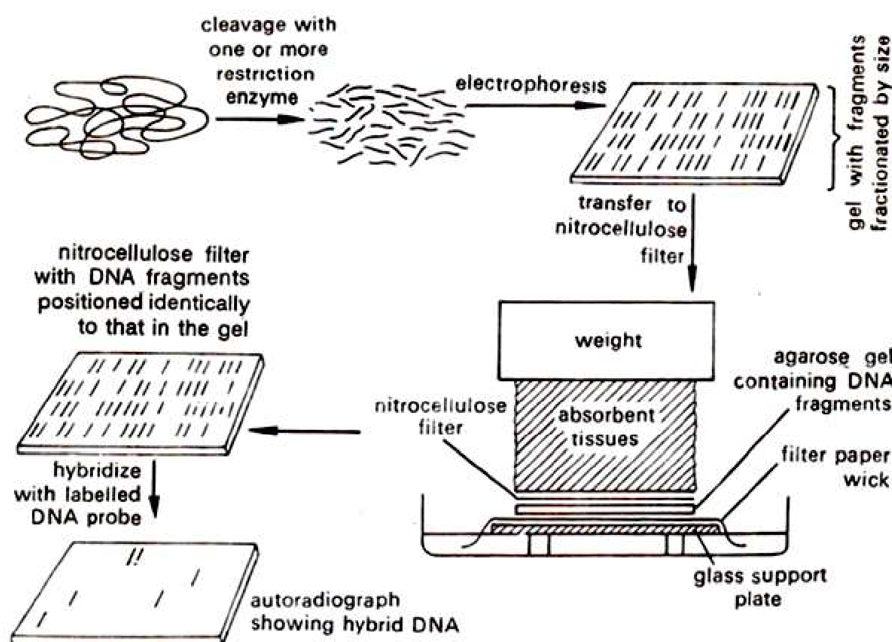


Fig. 8.5 *Southern Blotting Technique*

Advantages of Southern Blotting

Following information can be obtained from the technique of Southern blotting:

1. Whether a particular gene is present and how many copies are present in the genome of an organism.
2. The degree of similarity between the chromosomal gene and the probe sequence.
3. Whether recognition sites for particular restriction endonucleases are present in the gene. By performing the digestion with different endonucleases, or with combinations of endonucleases, it is possible to obtain a restriction map of the gene, i.e., an idea of restriction enzyme sites in and around the gene which will assist in attempts to clone the gene.
4. Whether rearrangements have occurred during the cloning process.

Applications of Southern Blotting

1. To identify a single gene among thousands of fragments of DNA and to detect sequences of DNA in an organism's genome.
2. Used in Gene Discovery and Gene Mapping.
3. To analyze the Genetic Patterns in an organism's DNA.
4. To identify Gene Mutation, Deletion, Duplication, and Gene Rearrangement involved in diseases.
5. To determine the number of copies of a particular DNA sequence presented in the Genome of an organism.

6. To identify Related DNA sequence in the Genome and to determine if there is a Gene Family, i.e., a group of similar genes.
7. To detect certain Cancers and Genetic Diseases, such as Monoclonal Leukemia population, Sickle Cell Mutation, used in DNA Fingerprinting, Genetic Engineering and Forensic Science for specific tests, such as paternity testing, personal identification, sex determination, and species exclusion.

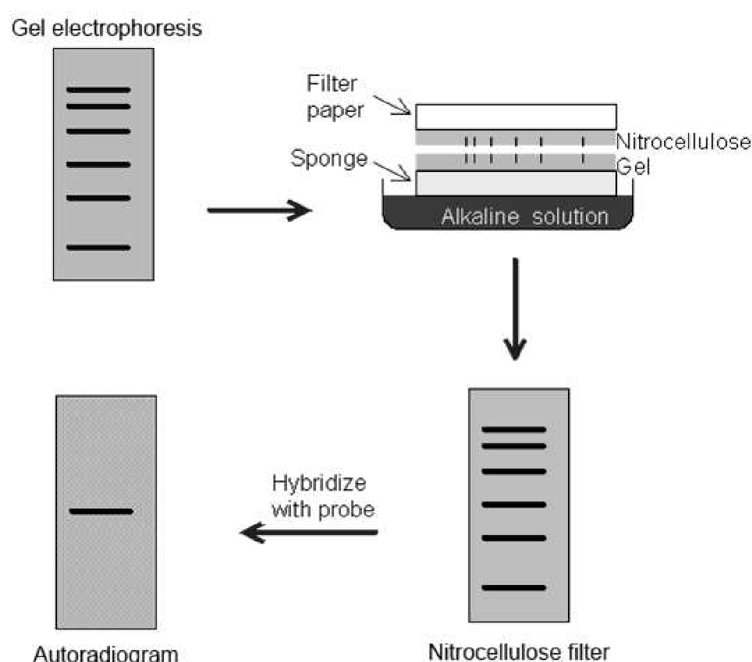


Fig. 8.6 *Steps of Southern Blotting*

Figure 8.6 illustrates the four significant steps of Southern blotting as follows:

Step 1: The DNA to be analyzed is digested with RE and separated by AGE.

Step 2: The DNA fragments in gel are denatured with alkaline solution and transferred onto a nitrocellulose filter by blotting.

Step 3: The nitrocellulose filter is incubated with a specific probe.

Step 4: The location of DNA fragment that hybridizes with probe can be displayed by autoradiography.

Northern Blotting

Northern blotting is a simple extension of Southern blotting, and derives its name from the earlier technique. It is one of the key techniques in molecular biology, its principal aim being the measurement of RNA (in particular mRNA). RNA molecules are separated by size and detected on a membrane using a hybridization probe with a base sequence complementary to all, or a part, of the sequence of target RNA.

RNA is extracted from the cells of interest, but precautions must be taken to avoid degradation of single-stranded RNA by RiboNuclease (RNase), which is found on the skin and on glassware. Wear gloves use specially treated plastics and glassware to avoid accidental introducing ribonuclease to extraction. Addition of Di-Ethyl-Pyro-Carbonate (DEPC) inhibits ribonuclease activity and also baking at high temperature destroys ribonuclease activity, only useful for treating heat resistant equipment like glassware. It is performed in following steps:

Step 1: RNA is isolated from several biological samples, for example various tissues, various developmental stages of same tissue, etc.

Step 2: The RNA samples are separated according to their size on an agarose gel.

Step 3: The gel is then blotted on a Nylon Membrane or a Nitrocellulose filter.

Step 4: The membrane is placed in a dish containing hybridization buffer with a labelled probe. RNA blots are most usually probed with cDNA fragments.

Step 5: The membrane is washed to remove unbound probe.

Step 6: The labelled probe is detected via autoradiography if a radioactive probe is used or via a chemoluminescence reaction if a chemically labelled probe is used. In both cases this results in the formation of a dark band on an X-ray film.

Figure 8.7 illustrates the basic principle of Northern blotting.

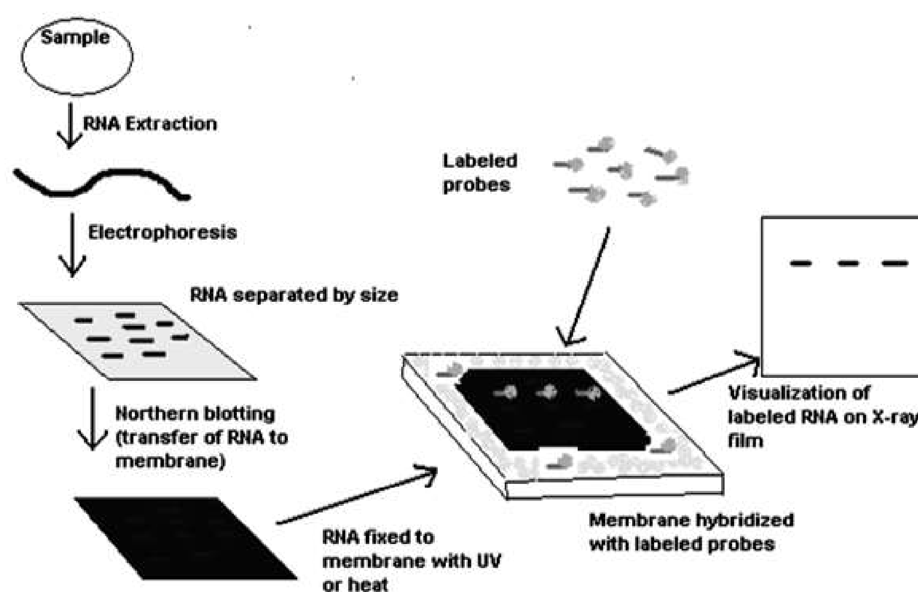


Fig. 8.7 Principle of Northern Blotting

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Advantages of Northern Blotting

Following information can be obtained from the technique of Southern blotting:

1. Differential expression patterns of a particular gene, such as:
   (a) In which tissues it is expressed.
   (b) If it is expressed during certain stages of development.
   (c) If expression changes under different conditions/treatments of the cell.
2. The quantity of mRNA present, blots can be quantified accurately by radioactive counting.
3. Whether a genomic DNA probe has regions that are transcribed.

Applications of Northern Blotting

1. Northern blotting allows researchers to determine gene expression patterns. This indicates a myriad of practical applications, allowing researchers to compare patterns of gene expression in cells of tissues, cells of patients undergoing treatment and cells of different developmental stages.
2. Northern blot analysis can also be used to detect Cancerous Pancreatic Cells and Tissues. In one study the researchers reviewed that the Pancreatic Cancers exhibited 3-fold, 10-fold and 15-fold increase in mRNA of a certain receptor, indicating for the first time that this receptor was involved in Carcinogenesis of Pancreatic Cancer. This information has been obtained by using Northern blotting technique.
3. Northern blotting can also enable the scientists to know the function of unknown Proteins.
4. This technique enables the scientists to detect the size of RNA.
5. It also allows them to observe the alternate splice products, using the probes with partial homology.

Western Blotting

The Western blot is an analytical technique used to detect specific Proteins in a given sample of tissue homogenate or extract. Sometime referred to as immune blotting, this technique uses gel electrophoresis to separate native or denatured Proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the Protein (native/non-denaturing conditions). Other related techniques include using antibodies to detect Proteins in tissues and cells by immune staining and Enzyme-Linked Immune Sorbent Assay (ELISA). This method originated from the laboratory of George Stark at Stanford. The name Western blot was given to the technique by W. Neal Burnette and is based on

the name Southern Blot, a technique for DNA detection developed earlier by Edwin Southern.

It is an analytical method wherein a Protein sample is electrophoresed on an SDS-PAGE and electro transferred onto nitrocellulose membrane. The transferred Protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate. In this technique first of all the sample of Proteins is separated on the basis of their molecular mass using SDS-PAGE or two-dimensional electrophoresis. Electrophoresis moves the Proteins from the gel and onto the nitrocellulose where Proteins adhere. To detect a specific Protein, an antibody to that Protein must be available. The Nitrocellulose Membrane itself has many non-specific sites that can bind Proteins, including antibodies which must be blocked with a non-specific Protein solution, such as rehydrated powdered milk. The primary antibody is added in the milk solution and binds to the Protein of interest. The antibody Protein complex is detected using a secondary antibody that has a label attached to it. Often a reporter enzyme, such as Alkaline Phosphatase is linked to the secondary antibody, and the addition of Lumiphos or X-Phos to the blot allows detection of the Protein Band.

Figure 8.8 illustrates the requirements and procedure of Western blotting.

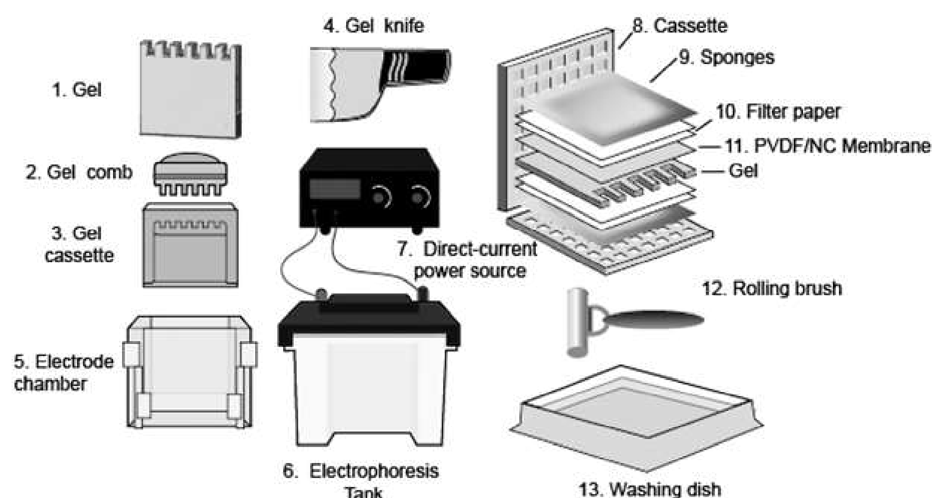


Fig. 8.8 *Requirements and Procedure of Western Blotting*

Advantages of Western Blotting

The Western blot provides the following information:

1. Size of Protein.
2. Expression Amount of Protein.

Applications of Western Blotting

1. Western blotting is mostly used as a medical diagnostic technique. A positive Western blot can usually confirm an HIV Infection. The confirmatory HIV test employs a Western blot to detect Anti-HIV antibody in a Human Serum sample.
2. A Western blot is also used as the definitive test for Bovine Spongiform Encephalopathy (BSE) commonly referred to as 'Mad Cow Disease'. Western blotting is also helpful in the diagnosis of some forms of Lyme disease.

Figure 8.9 illustrates the steps of Western blotting technique.

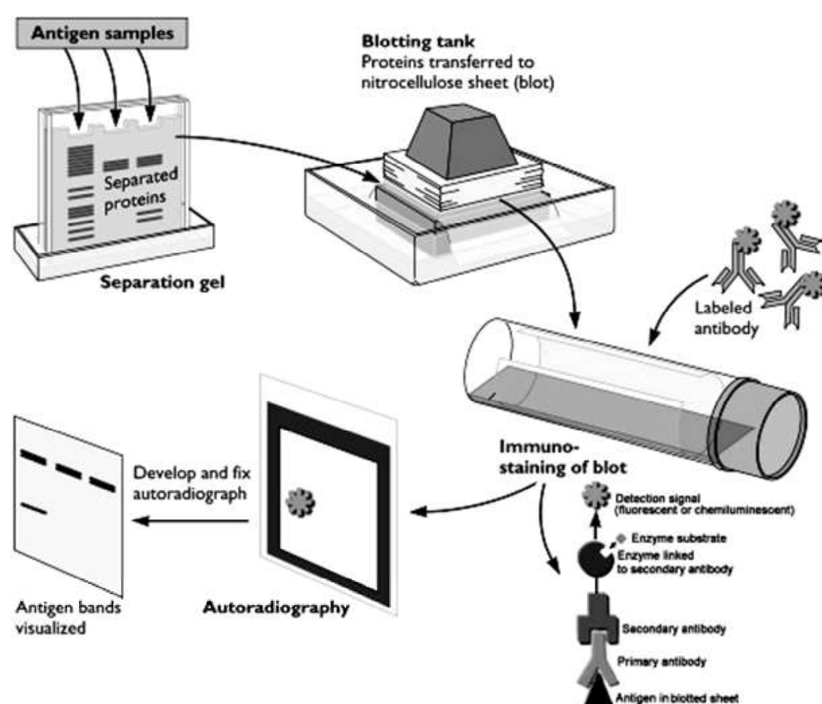


Fig. 8.9 *Steps of Western Blotting Technique*

Eastern Blotting

Eastern blotting is given by Bogdanov. This method is used to identify Carbohydrate Epitopes including Glycoconjugates and Lipids. Mostly blotted Proteins after transferring onto the membrane are analyzed for PTMs (Post-Translational Modification) by using a probe and hence identify Carbohydrates and Lipids. It involves the following steps:

Step 1: First, the targeted molecules are vertically separated by using gel electrophoresis.

Step 2: Then, these separated molecules are transferred horizontally on the Nitrocellulose membrane.

Step 3: After that primary antibody is added to the solution. These antibodies are responsible for recognizing a specific Amino Acid sequence. Then wash it to remove unbound primary antibody and add labeled secondary antibody. These labeled probes confirm the following molecules of interest:

- Detection of Protein modification.
- Used for binding studies by using various ligands.
- Used to purify various Phospholipids.

Figure 8.10 illustrates the principle of Eastern blotting.

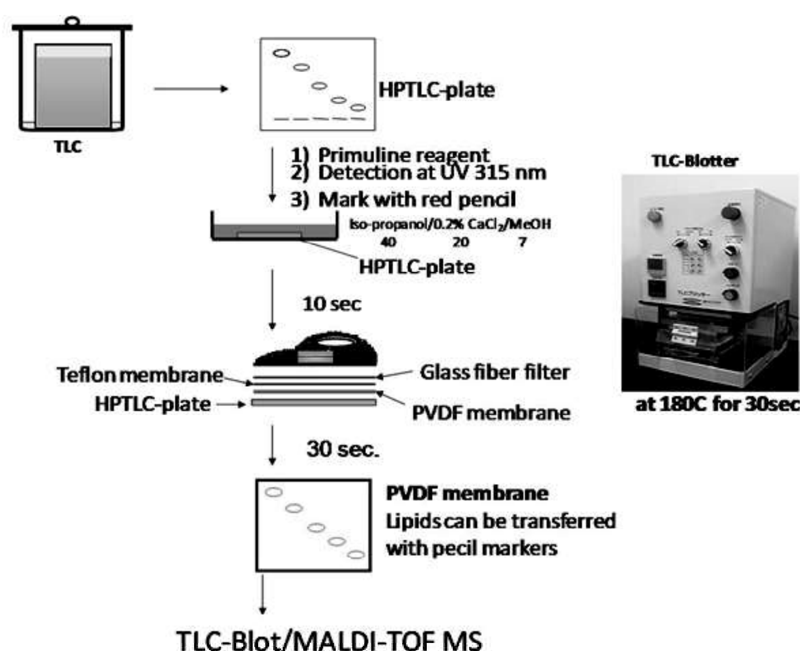


Fig. 8.10 *Principle of Eastern Blotting*

South-Western Blotting

South-Western blotting is the combination of Southern blotting, which was created by Edwin Southern, and Western blotting. This lab technique was first described by B. Bowen, J. Steinberg and colleagues in 1980 and was used to identify DNA-binding Proteins that specifically interact with a chosen DNA fragment in a sequence-specific manner. Thus it involves identifying and characterizing DNA-binding Proteins, i.e., the Proteins that bind to DNA by their ability to bind to specific oligonucleotide probes. The Proteins are separated by gel electrophoresis and are subsequently transferred to Nitrocellulose membranes, similar to other types of blotting. In this technique, mixtures of Proteins, such as

crude nuclear extracts or partially purified preparations are first fractionated on a Sodium Dodecyl Sulfate (SDS) denaturing gel. The gel is then equilibrated in a SDS-free buffer to remove detergent and the Proteins transferred by electro blotting to an immobilizing membrane. During the transfer, Proteins re-nature and hence DNA-binding Proteins may subsequently be detected on the membrane by their ability to bind radio-labeled DNA. Fractionation of crude nuclear extracts on an SDS gel followed by electro blotting and analysis for sequence-specific DNA binding directly on the blot combines the advantages of a high-resolution fractionation step with the ability to rapidly analyze for a large number of different DNA-binding specificities. Hence the name South-Western blotting is based on the fact that this technique detects DNA-binding Proteins, since DNA detection is done by Southern blotting and Protein detection is done by Western blotting.

Principle and Procedure of South-Western Blotting

South-Western blotting is a technique used to study DNA-Protein interactions. This method detects specific DNA-binding Proteins by incubating radiolabeled DNA with a gel blot, washing, and visualizing through autoradiography. A blot resulting from 1-dimensional SDS-PAGE reveals the molecular weight of the binding Proteins. To increase separation and determine isoelectric point a 2-dimensional gel can be blotted. Additional dimensions of electrophoresis, such as a gel shift (EMSA), can precede isoelectric focusing and SDS-PAGE to further improve separation. Combined with other techniques, such as mass spectrometry, the DNA-binding Protein can be identified.

'South-Western Blot Mapping' is performed for rapid characterization of both DNA-binding Proteins and their specific sites on genomic DNA. Proteins are separated on a PolyAcrylamide Gel Electrophoresis (PAGE) containing Sodium Dodecyl Sulfate (SDS), renatured by removing SDS in the presence of Urea, and blotted onto Nitrocellulose by diffusion. The genomic DNA region of interest is digested by restriction enzymes selected to produce fragments of appropriate but different sizes, which are subsequently end-labeled and allowed to bind to the separated Proteins. The specifically-bound DNA is eluted from each individual Protein-DNA complex and analyzed by polyacrylamide gel electrophoresis. Evidence that tissue-specific DNA binding Proteins may be detected by this technique has been presented. Moreover, their sequence-specific binding allows the purification of the corresponding selectively bound DNA fragments and may improve Protein-mediated cloning of DNA regulatory sequences.

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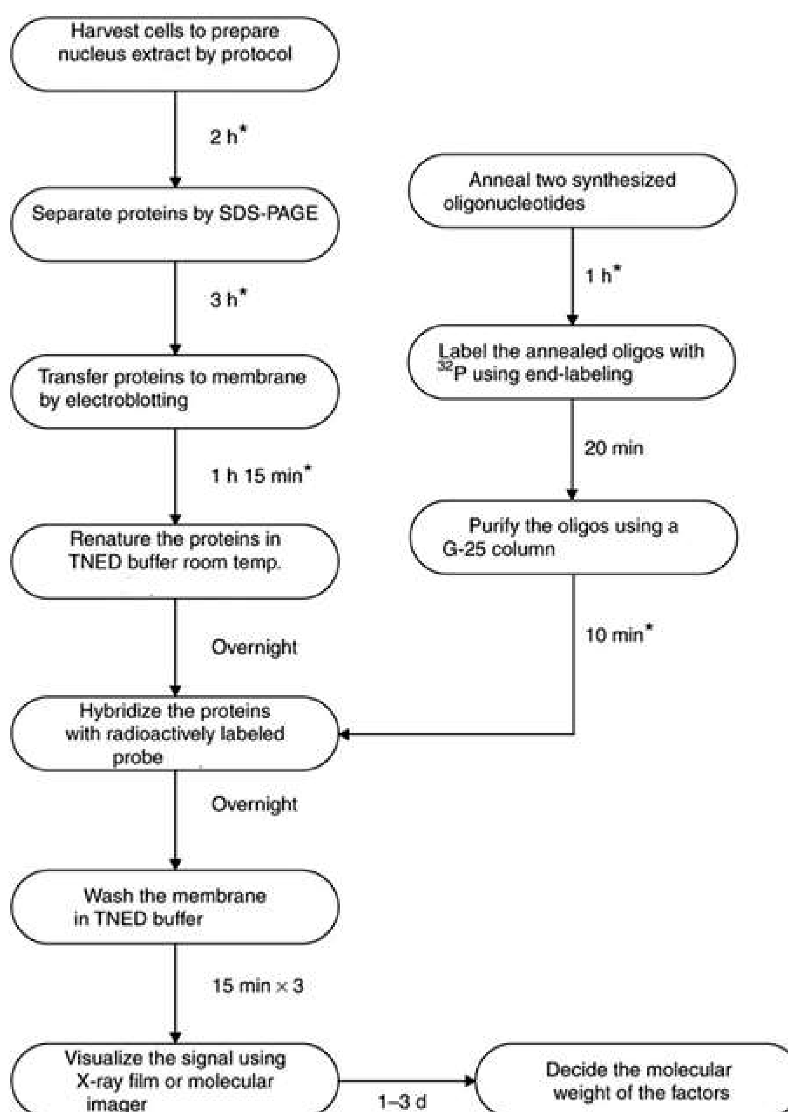


Fig. 8.11 Schematic Flow Chart of South-Western Blotting

Applications of South-Western Blotting

1. Identification of transcription factors specific to a particular gene is not only significant to gene regulation but also to understanding gene function. The South-Western Blotting (SWB) assay is one of the most powerful techniques to explore Protein-DNA interaction and transcription factor regulation.
2. SWB, similar to other blotting techniques, separates Proteins (or DNA) by gel electrophoresis. To detect the DNA-binding Proteins, the Proteins are partially renatured and bound to nanomolar concentrations of radiolabeled DNA, and then bands on the blot are detected by autoradiography. This technique is especially useful in the identification of transcription factor as it

gives information on the molecular weights of all DNA-binding Proteins involved with a particular sequence of DNA.

3. South-Western blotting is used to investigate DNA-Protein interactions. The advantage of this technique over other related methods, such as Electrophoretic Mobility Shift Assay (EMSA) and DNA foot-printing is that it provides information regarding the molecular weight of unknown Protein Factor.

North-Western Blotting

North-Western blotting technique is the combination of Northern blotting and Western blotting. This technique is used for identification of Protein-RNA interactions in which Protein is run on a gel, blotted, and probed with a labeled RNA of interest. Interactions are detected as hot-spots on the filter. Figure 8.12 illustrates the steps of North-Western blotting method.

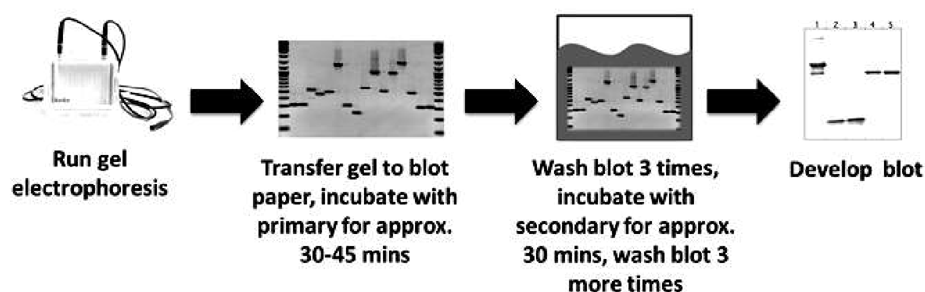


Fig. 8.12 *North-Western Blotting*

Zoo Blotting

Zoo blotting is based on the principles of Southern blotting. In the genome of any organisms there are two regions – the coding regions and the non-coding regions. It is the coding region that causes interest for most of the researchers as it is associated with the genetic information for a specific Protein. But the problem is that most of the regions of DNA are non-coding. The question is how to identify coding regions in large amount of non-coding DNA.

Zoo blotting is precisely used to distinguish coding DNA from non-coding regions. During evolution, the base sequence of non-coding DNA mutates and changes rapidly, whereas coding sequences change much more slowly and can still be recognized after millions of years of divergence between two species. Therefore, DNA is extracted from a series of related animals, such as a human, monkey, mouse, hamster, cow, etc. Samples in this DNA **'Zoo'** are each cut off with a suitable restriction enzyme and the fragments are run on a gel and transferred to a nylon membrane. They are probed using DNA that is suspected of being human coding DNA. Figure 8.13 illustrates the Zoo blotting method, here the probe is a segment of human DNA that may or may not be a coding region.

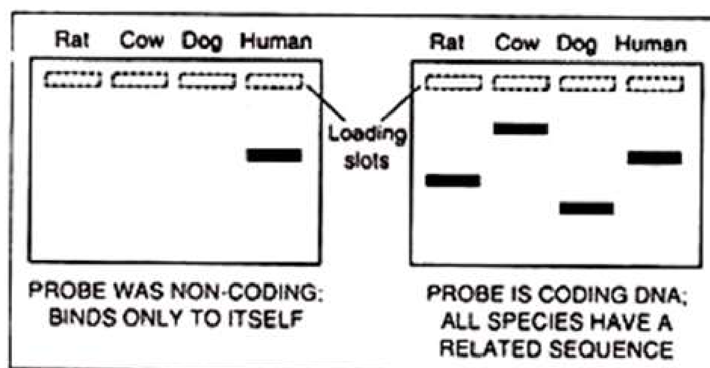


Fig. 8.13 *Zoo Blotting for the Probe Segment of Human DNA*

Dot Blotting

The dot blot is similar to the other blotting techniques, except it does not provide information regarding the size of the hybridized fragment. With this technique, extracted DNA or RNA from the target specimen is spotted onto the filter without the prior electrophoresis and transfer steps. In a reverse dot blot, it is the probe that is pre-bound to the filter and then hybridized with the patient's (usually PCR-amplified and colorimetrically tagged) DNA. The probe configuration may be as lines (line blot) rather than dots or circles.

Dot blotting is a modified version of Western blotting which is used for identification and analysis of Protein of interest. Dot blot methodology differs from traditional Western blot techniques by not separating Protein samples using electrophoresis. Sample Proteins are instead spotted onto membranes and hybridized with an antibody probe. Dot blot refers to the deposition of a Protein solution directly onto the membrane. If the volume to be added to the membrane is small (5 ml), the solution can be applied directly with a capillary micropipette. For larger volumes or when making quantitative measurements, dot blot or slot blot apparatuses are available that give uniform size dots or slots, therefore allowing the comparison of intensity of staining.

Since in the dot blot systems the molecular weight information is lost, this technique is especially well suited for probing a given antigen with different sera (as in the screening of monoclonal antibodies), or for quantization of a given Protein in a mixture by serial dilutions. Due to this limitation, dot blots can be used only if the specificity of the antibody is known, i.e., it reacts only with the antigen of interest. All the techniques for detection discussed above can be applied to a dotted membrane.

Principle for Qualitative Analysis by Dot Blotting

Dot blots are commonly used to probe for modified bases in gDNA. DNA is denatured to expose the bases, spotted onto an absorbent membrane, and probed with antibodies against each of the four cytosine modifications. Dot blots offer a

clear visual result and can be performed using either serial dilutions or single concentrations of DNA. We consider the former to be semi-quantitative, while the latter is only qualitative but still particularly useful for screening a large number of samples. Dot blotting also works for plasmids but is generally not well suited for short oligonucleotides, likely because these do not adhere consistently to membranes. The first step is to determine the appropriate amount of DNA for blotting, considering the amount of expected modifications. Finally expose on an imager with chemiluminiscent detection capabilities, taking care to smooth the blot over the imaging surface and remove air bubbles and excess substrate. As positive and negative controls for optimized protocol, typically uses gDNA from cells transfected or empty vector, respectively.

Figure 8.14 illustrates the representative dot blots from analysis of gDNA from transfected HEK293T cells. In Figure 8.14 (A) the serial dilutions of gDNA from cells transfected is shown with either empty expression vectors or hTET2-CD. In Figure 8.14 (B) the dot blots of gDNA from HEK293T cells transfected is shown with empty expression vector, hTET1-CD, hTET2-CD, or mTet2-CD.

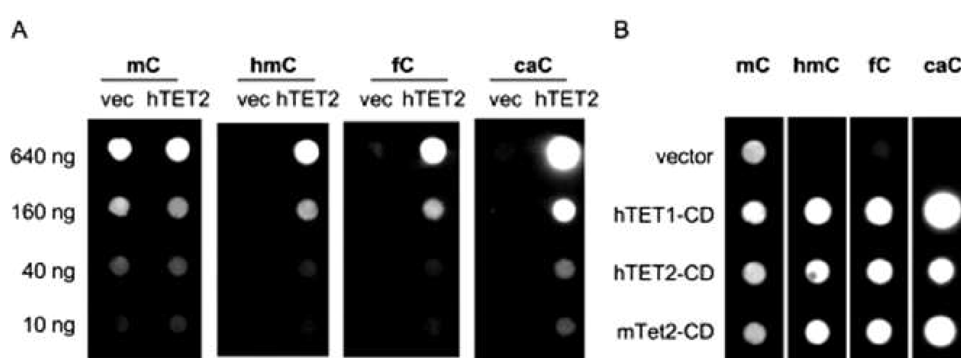


Fig. 8.14 *Representative Dot Blots from Analysis of gDNA from Transfected HEK293T Cells*

8.4 DOT AND SLOT BLOT HYBRIDIZATION

Dot and Slot Blotting are simple techniques for immobilizing bulk unfractionated DNA on a Nitrocellulose or Nylon Membrane. Hybridization analysis can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations. Dot and slot blots differ only in the geometry of the blot, a series of spots giving a hybridization pattern that is amenable to analysis by densitometric scanning. Samples are usually applied to the membrane using a manifold attached to a suction device. The basic protocol describes such a procedure for dot or slot blotting on an uncharged nylon membrane; annotations to the steps detail the minor modifications that are needed if blotting onto nitrocellulose. The first alternate protocol describes the more major changes required for blotting with a positively charged nylon membrane. A second alternate protocol describes preparation of dot blots by spotting the samples onto the

membrane by hand. Figure 8.15 illustrates the DNA Dot and Slot Blot Hybridization technique.

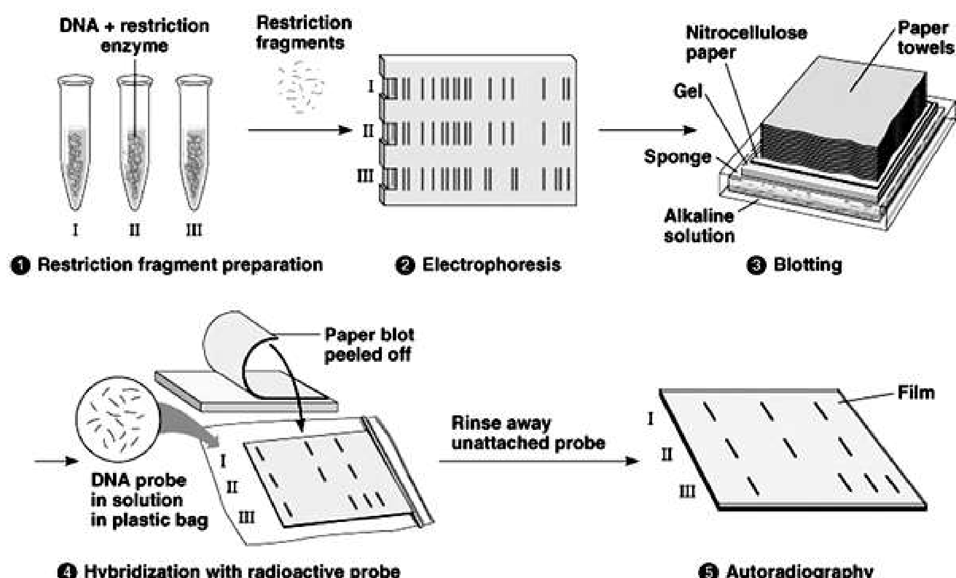


Fig. 8.15 DNA Dot and Slot Blot Hybridization Technique

Often it is informative to quantify the abundance of a certain RNA or DNA in the extracted nucleic acid mixture by dot blot or slot blot hybridization without prior digestion and electrophoresis. In the procedure, the nucleic acid mixture is blotted to a membrane where the hybridization is carried out. The difference between dot and slot blot procedures is in the way that the nucleic acid mixture is blotted onto the membrane. In dot blotting the nucleic acids are blotted as circular blots, whereas in slot blotting they are blotted into rectangular slots. The latter method allows a more precise observation of different hybridization signal intensities. Quantification of a certain RNA/DNA compared with total RNA/DNA can be obtained by hybridization with universal and specific oligonucleotide probes. The relative abundance is calculated by dividing the amount of specific probe bound to a given sample by the amount of hybridized universal probe measured, for example as fluorescence intensity (fluorescent probes) or counts per minute (radioactively labeled probes). Figure 8.16 illustrates the dots and slots in dot and slot blot hybridization, respectively.

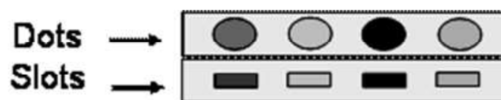


Fig. 8.16 Dots and Slots in Dot and Slot Blot Hybridization

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One should be aware that the data of relative DNA or RNA abundance cannot be directly translated into cell numbers. Cells may have different copy numbers of various genes. Moreover, cells of different species have different ribosome contents ranging roughly between 10^3 and 10^5 ribosomes per cell. Even for one strain, cellular rRNA contents can vary significantly (at least over one order of magnitude), since they are directly correlated with the growth rate. The relative rRNA abundance should, however, represent a reasonable measurement of the relative physiological activity of the respective population, since it is the product of the number of detected cells and the average rRNA content. This information on the general activity of a given population should not automatically be regarded as an indication of a specific kind of activity. Often, one population has the potential to catalyze different transformations - one genotype is linked to several phenotypes.

Check Your Progress

1. Define the isolation of nucleic acids.
2. Explain the three basic steps of isolating the genetic material.
3. Illustrate the isolation and purification of genomic DNA.
4. Elaborate on the isolation and purification of plasmid DNA.
5. What do you understand by the blotting techniques?
6. Illustrate the Southern blotting.
7. Explain the Northern blotting.
8. Define the Western blotting.
9. Interpret the Eastern blotting.
10. Elaborate on the dot blotting.

8.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.
2. Isolating the genetic material (DNA) from cells (Bacterial, Viral, Plant or Animal) involves three basic steps:
   - o Rupturing of cell membrane to release the cellular components and DNA,
   - o Separation of the nucleic acids from other cellular components,
   - o Purification of nucleic acids.

3. Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells.
4. This comprises growth of the bacterial cell, harvesting and lysis of the bacteria, and purification of the plasmid DNA. The growth of the bacterial cell involves growth of the bacterial cells in a media containing essential nutrients. The harvest and lysis of bacteria results in the precipitation of DNA and cellular proteins.
5. Blotting techniques were developed to discriminate fragments of nucleic acids. These techniques involve several processes; electrophoresis is one of the processes and is used to separate fragments of DNA and RNA.
6. Southern blot hybridization detects target DNA fragments that have been size-fractionated by gel electrophoresis. This technique was invented in 1975 by E.M. Southern. In this technique we exploit the property of a radio-labelled probe with the single stranded DNA.
7. Northern blotting is a simple extension of Southern blotting, and derives its name from the earlier technique. It is one of the key techniques in molecular biology, its principal aim being the measurement of RNA (in particular mRNA).
8. The Western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. Sometime referred to as immune blotting, this technique uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions).
9. Eastern blotting is given by Bogdanov. This method is used to identify Carbohydrate Epitopes including Glycoconjugates and Lipids. Mostly blotted proteins after transferring onto the membrane are analysed for PTMs (Post-Translational Modification) by using a probe and hence identify Carbohydrates and Lipids.
10. The dot blot is similar to the other blotting techniques, except it does not provide information regarding the size of the hybridized fragment. With this technique, extracted DNA or RNA from the target specimen is spotted onto the filter without the prior electrophoresis and transfer steps.

8.6 SUMMARY

- Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.
- Genomic DNA is found in the nucleus of all living cells with the structure of double-stranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells.
- DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells.
- The growth of the bacterial cell involves growth of the bacterial cells in a media containing essential nutrients. The harvest and lysis of bacteria results in the precipitation of DNA and cellular proteins.
- Blotting techniques were developed to discriminate fragments of nucleic acids. These techniques involve several processes; electrophoresis is one of the processes and is used to separate fragments of DNA and RNA.
- Southern blot hybridization detects target DNA fragments that have been size-fractionated by gel electrophoresis. This technique was invented in 1975 by E.M. Southern. In this technique we exploit the property of a radio-labelled probe with the single stranded DNA.
- Northern blotting is a simple extension of Southern blotting, and derives its name from the earlier technique. It is one of the key techniques in molecular biology, its principal aim being the measurement of RNA (in particular mRNA).
- The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. Sometime referred to as immune blotting, this technique uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions).
- Eastern blotting is given by Bogdanov. This method is used to identify Carbohydrate Epitopes including Glycoconjugates and Lipids.
- Mostly blotted proteins after transferring onto the membrane are analysed for PTMs (Post-Translational Modification) by using a probe and hence identify Carbohydrates and Lipids.

- The dot blot is similar to the other blotting techniques, except it does not provide information regarding the size of the hybridized fragment. With this technique, extracted DNA or RNA from the target specimen is spotted onto the filter without the prior electrophoresis and transfer steps.

8.7 KEY WORDS

- **Isolation of nucleic acids:** Isolation of nucleic acids is followed by quantitation of nucleic acids generally done by either spectrophotometric or by using fluorescent dyes to determine the average concentrations and purity of DNA or RNA present in a mixture.
- **Blotting techniques:** Blotting techniques were developed to discriminate fragments of nucleic acids. These techniques involve several processes; electrophoresis is one of the processes and is used to separate fragments of DNA and RNA.
- **Southern blotting:** Southern blot hybridization detects target DNA fragments that have been size-fractionated by gel electrophoresis. This technique was invented in 1975 by E.M. Southern.
- **Northern blotting:** Northern blotting is a simple extension of Southern blotting, and derives its name from the earlier technique. It is one of the key techniques in molecular biology, its principal aim being the measurement of RNA (in particular mRNA).
- **Western blotting:** The Western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract.
- **Eastern blotting:** Eastern blotting is given by Bogdanov. This method is used to identify Carbohydrate Epitopes including Glycoconjugates and Lipids.
- **Dot blotting:** The dot blot is similar to the other blotting techniques, except it does not provide information regarding the size of the hybridized fragment.

8.8 SELF-ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the isolation of nucleic acids.
2. Define the three basic steps of isolating the genetic material.
3. Interpret the isolation and purification of genomic DNA.
4. State the isolation and purification of plasmid DNA.

5. Elaborate on the blotting techniques.
6. Illustrate the Southern blotting.
7. State the Northern blotting.
8. Explain the Western blotting.
9. Define the Eastern blotting.
10. What do you understand by the dot blotting?

Long-Answer Questions

1. Discuss briefly the isolation and purification of genomic DNA.
2. Explain the isolation and purification of plasmid DNA. Write down the methods for separation of plasmid DNA.
3. Describe the Southern blotting with its advantages and applications.
4. Analyse the Northern blotting. What are the advantages and applications of Northern blotting?
5. Differentiate between the Western blotting and Eastern blotting.
6. Briefly define the principle and procedure of South-Western blotting.
7. What do you understand by the zoo blotting? Give appropriate examples.
8. Analyse the principle for qualitative analysis by dot blotting.
9. Explain the dot and slot blot hybridization with the help of examples.

8.9 FURTHER READINGS

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UNIT 9 METHODOLOGY OF DNA BARCODING

Structure

9.0 INTRODUCTION

DNA barcoding techniques were developed from early DNA sequencing work on microbial communities using the 5S rRNA gene. In 2003, definite techniques and terminology of modern DNA barcoding were proposed as a consistent way for identifying species, in addition to potentially assigning unknown sequences to higher taxa for instance- orders and phyla.

DNA barcoding refers to the method of species identification by using a short section of DNA from a specific gene or genes. The principle behind the methodology of DNA barcoding is that, by comparison with a reference database or library of such DNA sequences, an individual sequence can be used to uniquely identify an organism to species. These "Barcodes" are often used in an attempt to categorize unidentified species, parts of an organism, or merely to catalogue as many taxa as possible, or to compare with conventional or traditional taxonomy in an attempt to establish species boundaries.

Specific gene regions are used to identify the different organismal groups using the methodology of DNA barcoding. For instance, 16S rRNA gene is extensively used in identification of prokaryotes, cytochrome *c* oxidase I (COI or COX1) gene (present in mitochondrial DNA) is used as barcode region for animals, Internal Transcribed Spacer (ITS) rRNA and RuBisCO are regularly used for fungi and plants respectively. Whenever, the technique of DNA barcoding is used to categorize organisms from a study sample consisting DNA from several organism,

the term 'DNA Metabarcoding' is used to denote the process. For instance, DNA metabarcoding of water sample.

The reason behind picking these gene regions as barcodes is that they exhibit less intraspecific (within species) variation than interspecific (between species) variation, which is referred as the "Barcoding Gap".

In this unit, you will study about the DNA barcoding, principles, methods, and instrumentation of DNA sequencing, Maxam and Gilbert method, Sanger's di-deoxy method, and automated DNA sequencing.

9.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the PCR, RFLP, RAPD, and DNA finger printing
- Comprehend the DNA barcoding
- Define the principles, methods, and instrumentation of DNA sequencing
- Elaborate on the Maxam and Gilbert method
- Interpret the Sanger's di-deoxy method
- Analyse the automated DNA sequencing

9.2 METHODOLOGY OF DNA BARCODING

The following paragraph outlines the methodology of the DNA barcoding process:

Step 1: First step of the process involves isolating the DNA from the sample either individual, i.e., single species or mixed

Step 2: Second step of the process involves amplifying the target region, i.e., 'DNA Barcode' region via Polymerase Chain Reaction 'PCR'. The methodology of 'PCR' is as follows:

- The Polymerase Chain Reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis.
- This method is used in molecular biology to make millions of copies of (amplify) short sections of DNA or a gene.
- Five core 'Ingredients' are required to set up a PCR:- the DNA template to be copied, primers that initiate the PCR reaction, DNA bases (A, C, G and T), Taq polymerase enzyme to insert in the new DNA bases, DNA buffer to make sure the optimum conditions for the PCR reaction.
- PCR involves a repeated process of heating and cooling referred to as 'Thermal Cycling' which is carried out by PCR machine.

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- There are three main stages of a PCR reaction:
  1. **Denaturing** – This is first step of the process in which double-stranded template DNA is heated to 94-95p C which ensures the breaking of hydrogen bonds between the two strands and thus separating it into two single strands. This step usually takes between 15-30 seconds.
  2. **Annealing** – During this stage, temperature is lowered to 50-65p C to enable the DNA primers (short sequences of DNA 20 to 30 base in length) to attach to the template DNA. Cooling down help the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding. This step usually takes between 10 to 30 seconds.
  3. **Extending** – During this final step, the heat is increased to a temperature of 72p C to facilitate the new DNA to be made by a special Taq DNA polymerase enzyme which keeps on adding new DNA bases.
- All the above mentioned three steps are repeated 20-40 times, thus doubling the number of DNA copies every time.
- It usually takes a few hours to complete the entire PCR reaction
- The entire three step PCR reaction is summarized below:-

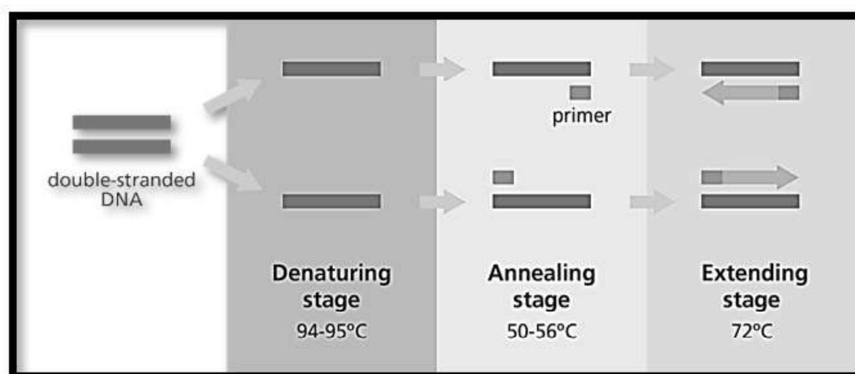


Fig. 9.1 *PCR Reaction*

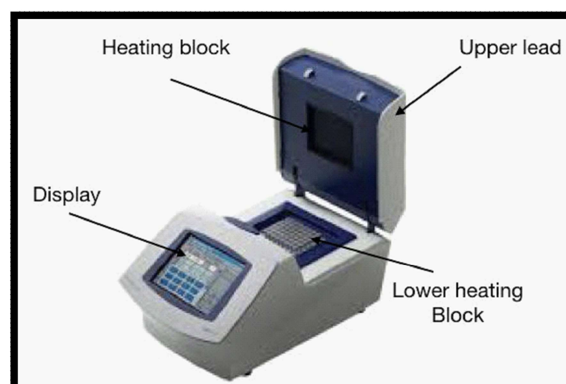


Fig. 9.2 *Parts of a PCR Machine*

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- After the PCR reaction has been completed, another methodology referred to as electrophoresis is used to check the size as well as quantity of amplified product.

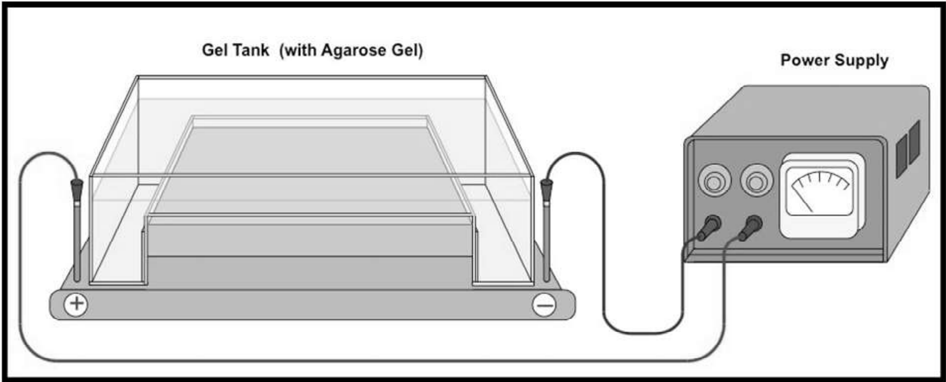


Fig. 9.3 *Agarose Gel Electrophoresis Apparatus*

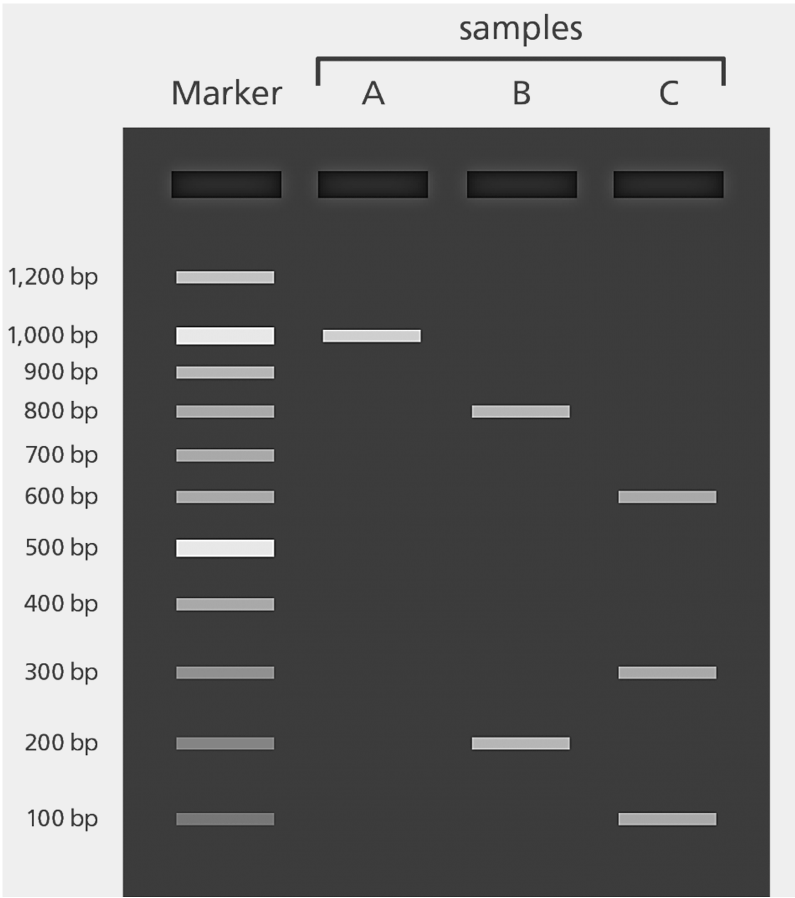


Fig. 9.4 *Agarose Gel Having DNA Markers of Different Sizes and DNA Samples Marked as A, B and C*

Step 3: The third step involves the sequencing of the amplified PCR products using methods such as Sanger sequencing.

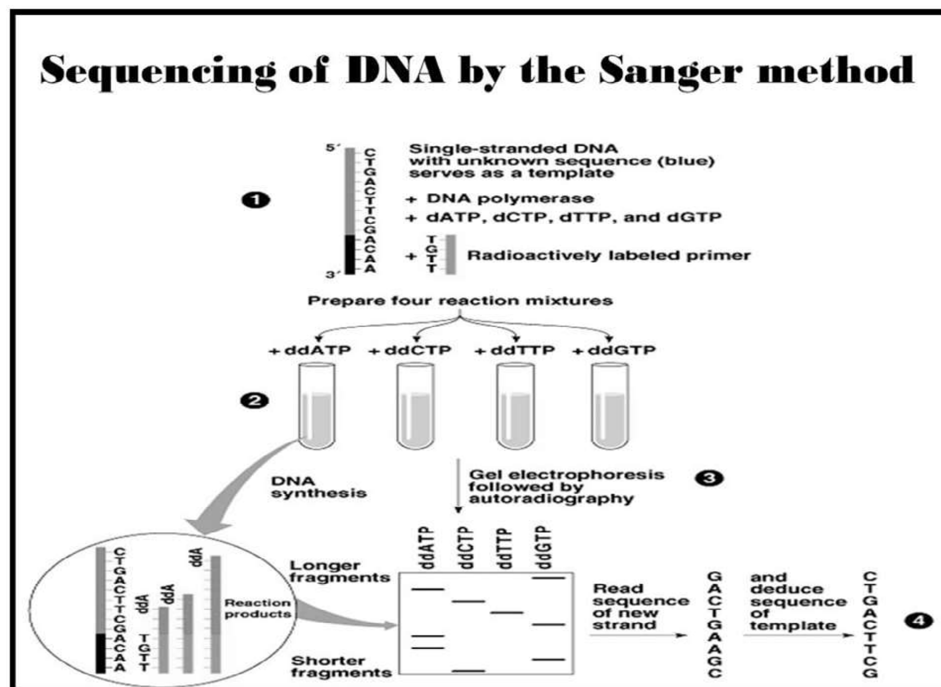


Fig. 9.5 *Overview of Sanger Sequencing*

Step 4: The last step of the process is to compare the resulting sequences against reference databases to find the matching species.

- BOLD is a searchable repository for barcode records, storing specimen data and images as well as sequences and trace files. It provides an identification engine based on the current barcode library and monitors the number of barcode sequence records and species coverage.
- Barcode sequences are positioned in the Barcode of Life Data Systems (BOLD) database – an online workbench that consists of a reference library of DNA barcodes that can then be used to allocate identities to sequences of unidentified origin.

NOTES

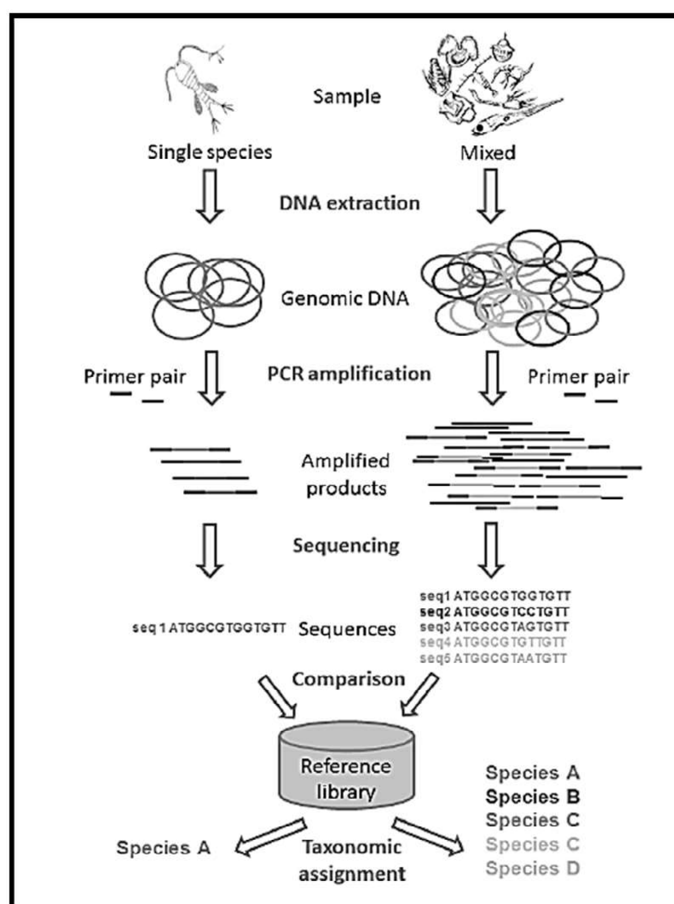


Fig. 9.6 *Overview of DNA Barcoding Methodology*



Fig. 9.7 *Screenshot of Bold Systems*

9.2.1 Application of DNA Barcoding

Researchers have applied this advanced DNA barcoding to deal with real-world problems having major impacts on every area where society interact with biodiversity such as:

- Resource management
- Research and development
- Production of food
- Food safety
- Management of pest population
- Education
- Biodiversity monitoring in terrestrial and aquatic environments
- Conservation of biodiversity
- Disease control
- Palaeontology and ancient ecosystems
- Plant-pollinator interactions
- Diet analysis
- Faecal analysis

9.2.2 RFLP, RAPD, and DNA Finger Printing

DNA profiling (also called DNA fingerprinting) is the process of determining an individual's DNA characteristics. DNA analysis intended to identify a species, rather than an individual, is called DNA barcoding.

DNA profiling is a forensic technique in criminal investigations, comparing criminal suspects' profiles to DNA evidence so as to assess the likelihood of their involvement in the crime. It is also used in parentage testing, to establish immigration eligibility, and in genealogical and medical research. DNA profiling has also been used in the study of animal and plant populations in the fields of zoology, botany, and agriculture.

RFLP

The first methods for finding out genetics used for DNA profiling involved RFLP analysis. DNA is collected from cells and cut into small pieces using a restriction enzyme (a restriction digest). This generates DNA fragments of differing sizes as a consequence of variations between DNA sequences of different individuals. The fragments are then separated on the basis of size using gel electrophoresis. The separated fragments are then transferred on to a nitrocellulose or nylon filter; this procedure is called a Southern blot. The DNA fragments within the blot are permanently fixed to the filter, and the DNA strands are denatured.

Radiolabeled probe molecules are then added that are complementary to sequences in the genome that contain repeat sequences. These repeat sequences tend to vary in length among different individuals and are called variable number tandem repeat sequences or VNTRs. The probe molecules hybridize to DNA fragments containing the repeat sequences and excess probe molecules are washed away. The blot is then exposed to an X-ray film. Fragments of DNA that have bound to the probe molecules appear as fluorescent bands on the film.

The Southern blot technique requires large amounts of non-degraded sample DNA. Also, Alec Jeffrey's original multilocus RFLP technique looked at many minisatellite loci at the same time, increasing the observed variability, but making it hard to discern individual alleles (and thereby precluding paternity testing). These early techniques have been supplanted by PCR-based assays.

RAPD

Random Amplification of Polymorphic DNA (RAPD), pronounced "Rapid", is a type of Polymerase Chain Reaction (PCR), but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8–12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from an RAPD reaction.

RAPD markers are decamer (10 nucleotides long) DNA fragments from PCR amplification of random segments of genomic DNA with a single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. It is used to analyse the genetic diversity of an individual by using random primers. Due to problems in experiment reproducibility, many scientific journals do not accept experiments merely based on RAPDs anymore. RAPD requires only one primer for amplification.

In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.

9.3 DNA BARCODES USED IN PROKARYOTES AND EUKARYOTES

There are several uses of DNA barcodes in prokaryotes and eukaryotes.

1. Plant barcoding studies involves using plastid regions such as rbcL and matK and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA.

NOTES



Fig. 9.8 *Plant Leave*

2. Animal barcoding studies use a region in the mitochondrial cytochrome *c* oxidase 1 gene ("CO1").



Fig. 9.9 *An Insect*

3. Fungal barcoding studies involve use of the Internal Transcribed Spacer (ITS) region in the nuclear ribosomal cistron. This ITS region exhibits sound discriminatory power at the species level in several groups.
4. 16S rRNA gene is extensively used in identification of prokaryotes.



Fig. 9.10 *Fungal Portion*

The reason behind picking these gene regions as barcodes is that they exhibit less intraspecific (within species) variation than interspecific (between species) variation, which is referred as the "Barcoding Gap".

NOTES

9.4 MAXAM AND GILBERT METHOD

Maxam–Gilbert sequencing is a method of DNA sequencing developed by Allan Maxam and Walter Gilbert in 1976–1977. This method is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides.

Maxam–Gilbert sequencing was the first widely adopted method for DNA sequencing, and, along with the Sanger di-deoxy method, represents the first generation of DNA sequencing methods. Maxam–Gilbert sequencing is no longer in widespread use, having been supplanted by next-generation sequencing methods.

Maxam–Gilbert sequencing requires radioactive labelling at one 52 end of the DNA fragment to be sequenced (typically by a kinase reaction using gamma-32P ATP) and purification of the DNA. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). For example, the purines (A+G) are depurinated using formic acid, the guanines (and to some extent the adenines) are methylated by dimethyl sulphate, and the pyrimidine's (C+T) are hydrolysed using hydrazine. The addition of salt (sodium chloride) to the hydrazine reaction inhibits the reaction of thymine for the C-only reaction. The modified DNAs may then be cleaved by hot piperidine; (CH2)5NH at the position of the modified base. The concentration of the modifying chemicals is controlled to introduce on average one modification per DNA molecule. Thus a series of labelled fragments is generated, from the radiolabeled end to the first "Cut" site in each molecule.

The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each showing the location of identical radiolabeled DNA molecules. From presence and absence of certain fragments the sequence may be inferred.

9.5 SANGER'S DI-DEOXY METHOD

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years. It was first commercialized by "Applied Biosystems" in 1986. More recently, higher volume Sanger sequencing has been replaced by "Next-Gen" sequencing methods, especially for large-scale, automated genome

analyses. However, the Sanger method remains in wide use, for smaller-scale projects, and for validation of Next-Gen results. It still has the advantage over short-read sequencing technologies (like Illumina) in that it can produce DNA sequence reads of > 500 nucleotides.

The classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotide triphosphates (dNTPs), and modified di-deoxynucleotide triphosphates (ddNTPs), the latter of which terminate DNA strand elongation. These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labelled for detection in automated sequencing machines.

Sanger methods achieve read lengths of approximately 800 bp (typically 500-600 bp with non-enriched DNA). The longer read lengths in Sanger methods display significant advantages over other sequencing methods especially in terms of sequencing repetitive regions of the genome. A challenge of short-read sequence data is particularly an issue in sequencing new genomes (de novo) and in sequencing highly rearranged genome segments, typically those seen of cancer genomes or in regions of chromosomes that exhibit structural variation.

9.6 AUTOMATED DNA SEQUENCING

The first DNA sequencing methods were developed by Gilbert (1973) and Sanger (1975). Gilbert introduced a sequencing method based on chemical modification of DNA followed by cleavage at specific bases whereas Sanger's technique is based on di-deoxynucleotide chain termination. The Sanger method became popular due to its increased efficiency and low radioactivity. The first automated DNA sequencer was the AB370A, introduced in 1986 by Applied Biosystems.

A DNA sequencer is a scientific instrument used to automate the DNA sequencing process. Given a sample of DNA, a DNA sequencer is used to determine the order of the four bases: G (guanine), C (cytosine), A (adenine) and T (thymine). This is then reported as a text string, called a read. Some DNA sequencers can be also considered optical instruments as they analyse light signals originating from fluorochromes attached to nucleotides.

The first automated DNA sequencer, invented by Lloyd M. Smith, was introduced by Applied Biosystems in 1987. It used the Sanger sequencing method, a technology which formed the basis of the "First Generation" of DNA sequencers and enabled the completion of the human genome project in 2001. This first generation of DNA sequencers are essentially automated electrophoresis systems that detect the migration of labelled DNA fragments. Therefore, these sequencers can also be used in the genotyping of genetic markers where only the length of a DNA fragment(s) needs to be determined (e.g. microsatellites, AFLPs).

NOTES

Automated DNA-sequencing instruments (DNA sequencers) can sequence up to 384 DNA samples in a single batch. Batch runs may occur up to 24 times a day. DNA sequencers separate strands by size (or length) using capillary electrophoresis, they detect and record dye fluorescence, and output data as fluorescent peak trace chromatograms. Sequencing reactions (thermo cycling and labelling), clean up and re-suspension of samples in a buffer solution are performed separately, before loading samples onto the sequencer. A number of commercial and non-commercial software packages can trim low-quality DNA traces automatically. These programs score the quality of each peak and remove low-quality base peaks (which are generally located at the ends of the sequence). The accuracy of such algorithms is inferior to visual examination by a human operator, but is adequate for automated processing of large sequence data sets.

Check Your Progress

1. Define DNA barcoding.
2. Who discovered the PCR reaction?
3. Explain the step denaturing of PCR reaction.
4. Illustrate the step annealing of PCR reaction.
5. What do you understand by the step extending?
6. Why some specific regions are used as DNA barcodes?
7. Explain the RFLP.
8. What is RAPD?
9. Interpret the agarose gel electrophoresis.
10. Elaborate on the Maxam–Gilbert method.
11. Illustrate the Sanger's di-deoxy method.

9.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. DNA barcoding refers to the method of species identification by using a short section of DNA from a specific gene or genes. The principle behind the methodology of DNA barcoding is that, by comparison with a reference database or library of such DNA sequences, an individual sequence can be used to uniquely identify an organism to species.
2. The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis.
3. Denaturing – This is first step of the process in which double-stranded template DNA is heated to 94-95p C which ensures the breaking of

hydrogen bonds between the two strands and thus separating it into two single strands. This step usually takes between 15-30 seconds.

4. Annealing – During this stage, temperature is lowered to 50-65p C to enable the DNA primers (short sequences of DNA 20 to 30 base in length) to attach to the template DNA. Cooling down help the primers to attach to a specific location on the single-stranded template DNA by way of hydrogen bonding. This step usually takes between 10 to 30 seconds.
5. Extending – During this final step, the heat is increased to a temperature of 72p C to facilitate the new DNA to be made by a special Taq DNA polymerase enzyme which keeps on adding new DNA bases.
6. The reason behind picking these gene regions as barcodes is that they exhibit less intraspecific (within species) variation than interspecific (between species) variation, which is referred as the "Barcoding Gap".
7. The first methods for finding out genetics used for DNA profiling involved RFLP analysis. DNA is collected from cells and cut into small pieces using a restriction enzyme (a restriction digest). This generates DNA fragments of differing sizes as a consequence of variations between DNA sequences of different individuals.
8. Random amplification of polymorphic DNA (RAPD), pronounced "Rapid", is a type of Polymerase Chain Reaction (PCR), but the segments of DNA that are amplified are random.
9. Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose.
10. Maxam–Gilbert sequencing is a method of DNA sequencing developed by Allan Maxam and Walter Gilbert in 1976–1977. This method is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides.
11. Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years.

9.8 SUMMARY

- DNA barcoding refers to the method of species identification by using a short section of DNA from a specific gene or genes. The principle behind the methodology of DNA barcoding is that, by comparison with a reference

database or library of such DNA sequences, an individual sequence can be used to uniquely identify an organism to species.

- The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis.
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- The first methods for finding out genetics used for DNA profiling involved RFLP analysis. DNA is collected from cells and cut into small pieces using a restriction enzyme (a restriction digest).
- Random Amplification of Polymorphic DNA (RAPD), pronounced "Rapid", is a type of Polymerase Chain Reaction (PCR), but the segments of DNA that are amplified are random.
- Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose.
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- Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating di-deoxynucleotides by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years.
- The first DNA sequencing methods were developed by Gilbert (1973) and Sanger (1975). Gilbert introduced a sequencing method based on chemical modification of DNA followed by cleavage at specific bases whereas Sanger's technique is based on di-deoxynucleotide chain termination.
- The first automated DNA sequencer, invented by Lloyd M. Smith, was introduced by Applied Biosystems in 1987. It used the Sanger sequencing method, a technology which formed the basis of the "First Generation" of

DNA sequencers and enabled the completion of the human genome project in 2001.

9.9 KEY WORDS

- **DNA barcoding:** DNA barcoding refers to the method of species identification by using a short section of DNA from a specific gene or genes.
- **Denaturing:** This is first step of the process in which double-stranded template DNA is heated to 94-95p C which ensures the breaking of hydrogen bonds between the two strands and thus separating it into two single strands.
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- **Extending:** During this final step, the heat is increased to a temperature of 72p C to facilitate the new DNA to be made by a special Taq DNA polymerase enzyme which keeps on adding new DNA bases.
- **Maxam–Gilbert method:** This is a method of DNA sequencing developed by Allan Maxam and Walter Gilbert in 1976–1977. This method is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides.
- **Sanger's method:** Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating di-deoxynucleotides by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years.
- **Automated DNA sequencer:** The first automated DNA sequencer, invented by Lloyd M. Smith, was introduced by Applied Biosystems in 1987.

9.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the DNA barcoding.
2. Define the step denaturing of PCR reaction.
3. Interpret the step annealing of PCR reaction.
4. What do you mean by the step extending?

5. Elaborate on the RFLP.
6. Illustrate the term RAPD.
7. State the agarose gel electrophoresis.
8. Define the Maxam–Gilbert method.
9. Explain the Sanger's di-deoxy method.

Long-Answer Questions

1. Discuss the process of DNA barcoding in detail.
2. Briefly describe the process of PCR.
3. Write down the DNA barcodes used in bacteria, fungi, plants, and animals.
4. Explain the applications of the process DNA barcoding.
5. Analyse the Maxam–Gilbert method.
6. Describe the Sanger's di-deoxy method.
7. Discuss briefly the automated DNA sequencing with the help of examples.

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UNIT 10 PHEROMONES IN PEST MANAGEMENT

NOTES

Structure

10.0 INTRODUCTION

Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other.

The composition of the pheromones may be saturated carbonic acid, steroids, aldehydes, ketones, alcohols or other compounds. Insects uses pheromones as chemical signals to search food, attract mate or warn others of predators. By utilizing particular pheromones, traps can be used to observe or monitor the target pests in agriculture as well as in residential areas.

This could lessen damage to agriculture crops, plants as well as damage caused to the residential and commercial structures. This can also limit the presence of stinging insects in the nearby residential areas. Pheromones can also help to find locations where the pests are becoming established. For instance- Insect pests like Asian gypsy moths and Japanese beetles can be damaging to plants and thus can be conveniently limited with the help of such community traps.

The portmanteau word "Pheromone" was coined by Peter Karlson and Martin Lüscher in 1959, based on the Greek pheroo ('I carry') and hormone ('Stimulating'). Pheromones are also sometimes classified as ecto-hormones. They were researched earlier by various scientists, including Jean-Henri Fabre, Joseph A. Lintner, Adolf Butenandt, and ethologist Karl von Frisch who called them various names, like for instance "Alarm Substances". These chemical messengers are transported outside of the body and affect neurocircuits, including the autonomous

nervous system with hormone or cytokine mediated physiological changes, inflammatory signalling, immune system changes and/or behavioural change in the recipient.

NOTES

In this unit, you will study about the pheromones in pest management, insect and rodent control, pheromones in animal breeding, conservation and management of indigenous cow, buffalo, tiger, and elephant.

10.1 OBJECTIVES

After going through this unit, you will be able to:

- Explain the uses of pheromones in pest management, insect and rodent control
- Define the applications of pheromones in animal breeding
- Analyse the concept of conservation and management of indigenous cow, buffalo, tiger, and elephant

10.2 PHEROMONES IN PEST MANAGEMENT - INSECT CONTROL

Pheromones traps: - A pheromone trap is used to capture the insect pest using specific pheromones. To ensure the effectiveness of pheromone traps:-

- Ascertain the quality of lure used for trap.
- Ascertain the quality as well as the specificity of the pheromone.
- Installation of the trap should be done at right time.
- Installation of the trap should be done at right place, i.e., stick/log.
- Installation of the trap should be done at proper height.
- Proper care of the polythene sleeve should be taken.
- Damaged sleeves should be replaced immediately.
- Proper distance should be maintained between the traps.
- Pheromone lure should be replaced timely for maintaining the effectiveness of the pheromone trap.
- Different types Pheromones- Lures used are: - Fibre, Plastic Tube, Rubber Septa, Controlled Release Membrane, Beads, Oil Solutions, Gels, etc.
- Different types of pheromone traps used are:- Funnel Traps, Grain Probe Traps, Pitfall Traps, Sticky Traps, Box Traps, Wing Traps, Delta Traps, Diamond Traps and Discreet Trap.

NOTES

Insect	Name of Pheromone	Chemical structure
Bombyx mori	Bombykol	10,12 Hexa-decadien 1-ol
Porthetria dispar	Gyplure	1 Hexal-12-Hydroxy-3 Dodisenile acetate
Honey bee(*Apis spp.*)	Queen substance	9.0x0-trans-2-Decenoic acid
Periplanata americana	----	2,2 Dimethyl-3-iso propelidine cyclo proply propionate
Mad fly	Singlure	2,3 secondary, Butyle 4 chloro 2 methyl hexane
Mad fly	Trimedlure	2,3 Ter.Butyl.
Oriental fruit fly	Methyl eugenol	1 Alil 1,2-Diemethoxy Benzene.
Carda cautella	---	9,12 tetra decadien 1- ol acetate
Pectinophora gossypiella	---	10-prophy-trans 5,9 tridecadien 1-ol-acetate
Porthetria dispar	---	D-10-acetoxy-cis 7-hexa decen-1-ol.

Fig. 10.1 *Chemical Structure of Sex Hormones Released by Different Insects*

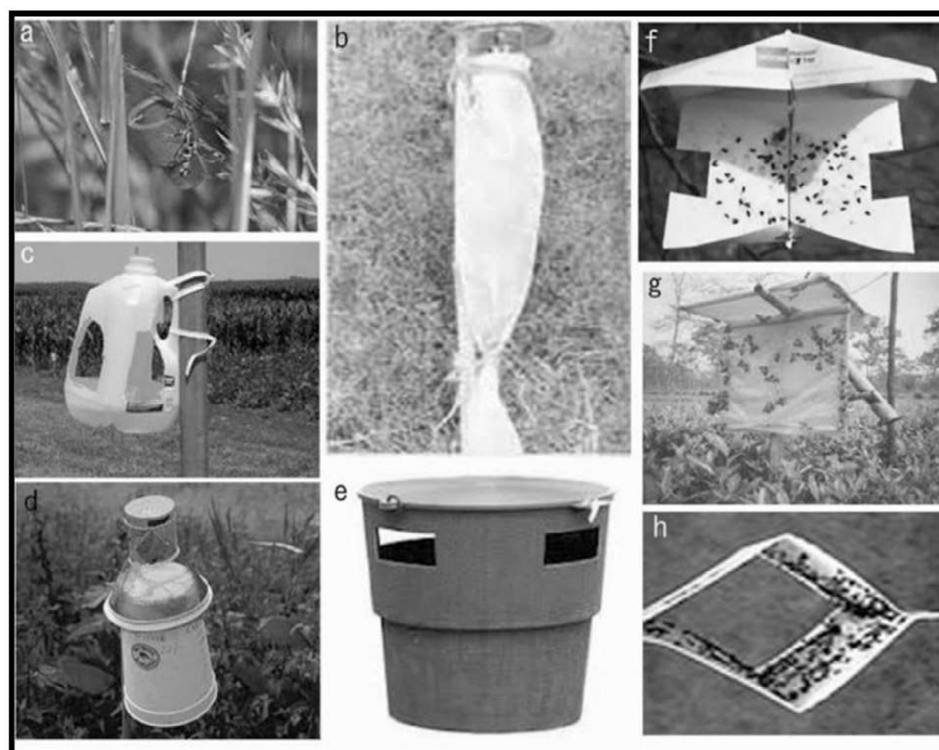


Fig. 10.2 *Different Types of Pheromones Traps. (a) One Lure Septum Hanged with Crop Plant, (b) Net Trap, (c) Water Trap, (d) Bucket Trap, (e) Bucket with Window Trap, (f) Delta Trap, (g) Yellow Sticky Trap, (h) Wing Trap*

NOTES

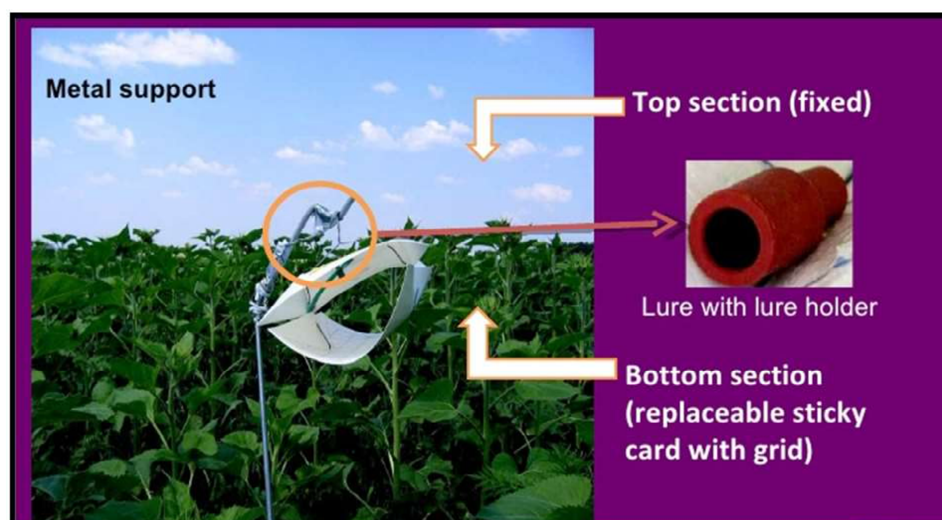


Fig. 10.3 *Parts of a Pheromone Trap*

10.3 PHEROMONES IN PEST MANAGEMENT - RODENT CONTROL

When compared to the success rate of pheromones in regulating insects pest populations, the far more complex behaviour of rodents constitutes a hindrance to the study of pheromonal effects and the practical use of pheromones in rodent control. However, pheromones do play an important role in rodents. In rodents, pheromones can be broadly divided into two major groups namely:

1. Signal pheromones or releasers, i.e., substances that trigger immediate behavioural responses
2. Primers, i.e., substances that produce effects that become manifest only after some time has passed, by working through some neuroendocrine pathway.

Releaser (signal) pheromones seem to be more promising in rodent control. These pheromones act as sex attractants as well as aggression eliciting pheromones. The sex pheromone which is found in female rodent urine or vaginal discharge has been used successfully as an important substance for attracting males to traps or to stations with toxic bait or chemosterilants. Further, a male produced odour attracting females could be very useful in rodent control. For instance, preputial glands in mice produce a specific factor that serve as a strong attractant to sexually active females.

10.4 CLASSIFICATION OF PHEROMONES

NOTES

Pheromones can be classified into following types on the basis of their function:

1. Aggregation Pheromones

- As the name suggests, aggregation pheromones are used to gather together individuals of the same species both males as well as females in moderately large numbers. Thus, aggregation pheromones are used by an organism for task that requires collective efforts such as invading a new habitat.
- These pheromones also perform other functions such as mate selection, overcoming host resistance by mass attack as well as defence against predators.
- Aggregation pheromones are one of the most ecologically selective pest suppression methods.

2. Alarm Pheromones

- Alarm pheromones can trigger flight or aggression in individuals of the same species.
- Alarm pheromones are also used to warn members of the same species about upcoming danger.
- For instance, aphids uses an alarm pheromone to inform other individuals of their species that they were attacked by a predator like lady bug.
- Some plants when grazed upon, results in tannin production in neighbouring plants which make the plants less appetizing for the herbivore.

3. Releaser Pheromones

- Releaser pheromones are pheromones that cause an alteration in the behaviour of the recipient.
- Releaser pheromone leads to rapid response, however, it is quickly degraded.
- For instance, a few organisms use potent attractant molecules to attract mates from a very large distance.

4. Signal Pheromones

- Signal pheromones leads to short-term changes like neurotransmitter.

5. Primer Pheromones

- Primer pheromones trigger a change of developmental events whereas other pheromones leads to a change in behaviour.

6. Territorial Pheromones

- Territorial pheromones mark the boundaries of an organism's territory.
- In some animals like cats and dogs, these pheromones are present in the urine and are used to mark the perimeter of the claimed territory.

7. Trail Pheromones

- Trail pheromones are common in social insects, such as ants, honeybees, etc.
- For instance, ants mark their paths with trail pheromones so that they can follow each other. This trail attracts other ants and serves as a guide to food source for them.

8. Sex Pheromones

- Sex pheromones are involved in the process of reproduction by attracting the opposite sex in animals.
- Release of sex pheromones indicates the availability of the female for breeding.
- Sometimes male animals also emit pheromones that transmit information about their species and genotype.
- Pheromones released by female are used to attract male of the same species.
- Pheromones released by males are used to attract female of the same species.

9. Host-Marking Pheromone

- Host marking pheromones are generally used by parasitoids to avoid ovipositing on hosts.
- Hyper parasitoids may use these host marking pheromones to look for their hosts.

10.5 ADVANTAGES AND DISADVANTAGES OF USING PHEROMONES IN PEST CONTROL

There are several advantages of using pheromones:

- Pheromones helps in controlling urban pests.
- Pheromones helps in monitoring exotic pests.

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- Pheromones helps in mass trapping of insects from breeding and feeding potential.
- Pheromones helps in disrupting mating of insect population.
- Very minute quantity of pheromone is required for making pheromones traps.
- Pheromones are non-polluting and ecologically acceptable.
- Pheromones are species specific.
- Helps in easy monitoring of pest population.
- Pheromones are best suited in Integrated Pest Management (IPM) techniques.
- Pheromones are quite cost effective and labour saving.

Disadvantages of using Pheromones in Pest Control

- Pheromones are yet to be identified in several wild species.
- Sex pheromones are capable of attracting only one sex.
- Farmers are not well equipped with the use of pheromones.
- Quick results cannot be obtained with application of pheromones.

10.6 PHEROMONES IN ANIMAL BREEDING

Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other. Pheromones are majorly used in cattle and pig breeding. They are sold as commercial preparations in the market based on their structural analogues. However, in some farms, nasal rings are used with substances containing pheromones.

These substances are urine and mucus from cows at the height of their estrous cycle. Pheromones play an essential role in the initiation of a particular behaviour or affecting the reproductive physiology of the animal by altering the activity levels of the internal hormones. Due to this reason, pheromones are also considered as ectohormones, i.e., chemical messengers that are transmitted outside the body. They support or increase the attractiveness of opposite poles as well as promotes aggression between males, accelerate the attainment of puberty, shortens the period of anoestrus, brings about changes in the estrous cycle and also plays an essential role in inducing mating behaviour in both sexes.

Additionally, biostimulatory effects of pheromones have been observed in domesticated as well as farm animals in terms of positive effect of the presence of breeding bulls on ovarian activity in cows and the success or failure of the

conception. An increase in the ejaculate volume as well as the number of live, health and motile sperms have been observed after nasal spray application of pheromones in farm animals. Thus, from the above discussion, we conclude that pheromones not only play a significant role in natural breeding process but also in artificial breeding technologies like artificial insemination and MOET or ETT technology discussed below.

A. Artificial Insemination

Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female. There are three common methods of Artificial Insemination (AI):

(i) Vaginal method

(ii) Recto vaginal method

(iii) The speculum method

Based on the size as well as the structure of the bovine reproductive tract, two methods namely Recto vaginal method and the speculum method are widely accepted as suitable methods for insemination in beef cattle. Below, we will discuss both the methods in detail:

(a) Recto Vaginal Method- This is one of the most widely accepted methodology in dairy industry due to its practicality as well as it is believed to be the safest for the animal. This methodology involves the manipulation of the reproductive tract, primarily the cervix, through rectal palpation.

(b) The Speculum Method- This methodology involves placing a speculum (or spectrum) into the vagina pushed up to the posterior end of the cervix.

Benefits of Artificial Insemination

- Increase in efficiency of usage of genetically superior bull,
- Increased potential for genetic selection,
- Cheap and economical over a long period of time,
- Protecting the interest of both dairy farmers as well as animals,
- Reduction in the transmission of diseases.

Drawbacks of Artificial Insemination

- More laborious technology requires skilled labour,
- Dairy farmer needs to detect the most genetically fit male to carry out the process of Artificial insemination,
- It can decrease the genetic variability over a long period of time.

B. Embryo Transfer Technology (ETT)

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The first Embryo Transfer Technology (ETT) project in the country was initiated by NDDB (National Dairy Development Board) in 1987 by the establishment of a central ET laboratory at Sabarmati Ashram Gaushala (SAG), Bidaj. The project was funded by the Department of Biotechnology (DBT), Ministry of Science & Technology, GoI for 5 years i.e. from April 1987 to March 1992. Under this project, NDDB established one Main ET Lab at SAG Bidaj and four Regional ET Labs at CFSP&TI, Hessarghatta (Karnataka), ABC, Salon (UP), Shri Nashik Panchavati Panjrapole, Nashik (Maharashtra) and Buffalo Breeding Centre, Nekarikallu (AP). NDDB also assisted in establishment of 14 State ET centres across the country.

'Embryo Transfer' (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals. Usually, one can get one calf from a superior quality female dairy animal in a year. However, by using 'MOET' /'ET' technology, dairy workers can get 10-20 calves in a year from a cow/buffalo. A genetically superior cow/buffalo is administered artificial hormones with FSH-like activity to induce super-ovulation. Under the influence of the FSH-like hormone, the genetically superior female produces numerous eggs instead of single egg produced in general. The super-ovulated female is artificially inseminated multiple time (2-3 time) at 12 hour interval during estrus and on 7th day post insemination the uterus of the artificially inseminated female is flushed with a medium to recover the developing embryos. Embryos are collected along with flushing medium in a specific filter. Thereafter, the quality of the developing embryos is assessed under the microscope. Superior quality embryos are either preserved or frozen for the purpose of transfer in future or they are transferred into recipient females (surrogate mother) roughly seven days post the heat date. Thus, with the use of 'ET' technology numerous genetically superior calves can be produced in a year.

SAG has done ground-breaking work in this field and so far has produced 14,388 viable embryos and 755 calves, which is maximum by any organisation in the country. Of these, 1026 embryos are of indigenous cattle breeds, from which 122 calves have been born. Besides these, around 3000 embryos of buffalo breeds have also been produced. Under the project, the first buffalo calf of India from frozen thawed embryo was born in the year 1991.

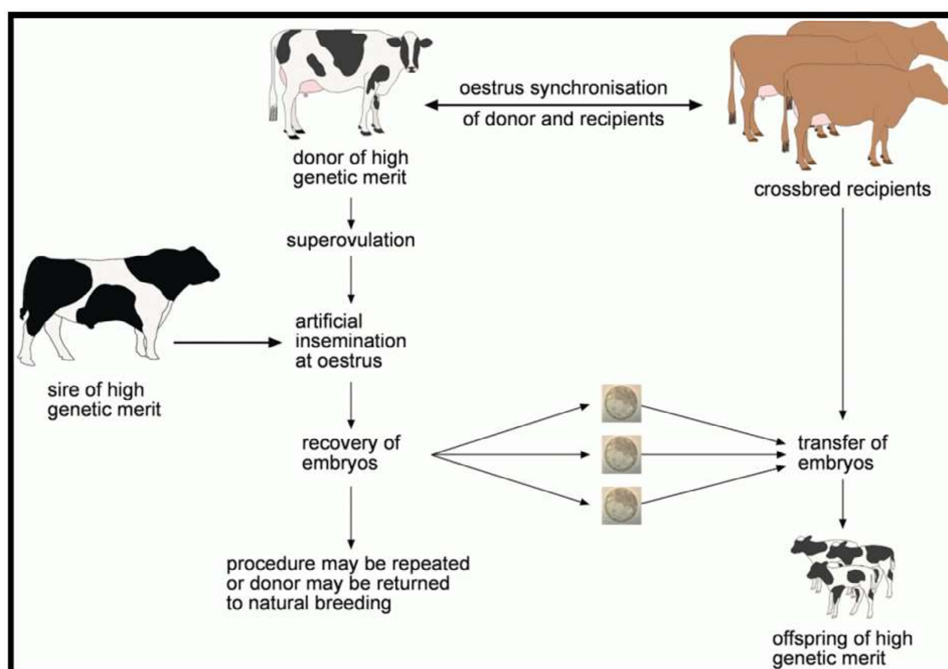


Fig. 10.4 *Embryo Transfer Technology*

Check Your Progress

1. Elaborate on the pheromones.
2. What is a pheromone trap?
3. Define the term aggregate pheromones.
4. Illustrate the artificial insemination.
5. Explain the embryo transfer technology.

10.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other. The composition of the pheromones may be saturated carbonic acid, steroids, aldehydes, ketones, alcohols or other compounds.
2. Pheromones traps: - A pheromone trap is used to capture the pest using specific pheromones.

3. Aggregation Pheromones- As the name suggests, aggregation pheromones are used to gather together individuals of the same species both males as well as females in moderately large numbers. Aggregation pheromones are used by an organism for task that requires collective efforts such as invading a new habitat. Aggregate pheromones also perform other functions such as mate selection, overcoming host resistance by mass attack as well as defence against predators. Aggregation pheromones are one of the most ecologically selective pest suppression methods.
4. Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female. There are three common methods of Artificial Insemination (AI):- Vaginal method, recto vaginal method and the speculum method.
5. Embryo Transfer (ET) also referred to as 'Multiple Ovulation and Embryo Transfer' (MOET) technology, is used to augment the reproduction rate of genetically superior female dairy animals. Usually, one can get one calf from a superior quality female dairy animal in a year. However, by using 'MOET /ET' technology, dairy workers can get 10-20 calves in a year from a cow/ buffalo.

10.8 SUMMARY

- Pheromones traps: - A pheromone trap is used to capture the insect pest using specific pheromones.
- Different types of pheromone traps used are:- Funnel Traps, Grain Probe Traps, Pitfall Traps, Sticky Traps, Box Traps, Wing Traps, Delta Traps, Diamond Traps and Discreet Trap.
- When compared to the success rate of pheromones in regulating insects pest populations, the far more complex behaviour of rodents constitutes a hindrance to the study of pheromonal effects and the practical use of pheromones in rodent control. However, pheromones do play an important role in rodents.
- The sex pheromone which is found in female rodent urine or vaginal discharge has been used successfully as an important substance for attracting males to traps or to stations with toxic bait or chemosterilants. Further, a male produced odour attracting females could be very useful in rodent control.
- Aggregation pheromones are used to gather together individuals of the same species both males as well as females in moderately large numbers.

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- Alarm pheromones can trigger flight or aggression in individuals of the same species. Alarm pheromones are also used to warn members of the same species about upcoming danger.
- Releaser pheromones are pheromones that cause an alteration in the behaviour of the recipient. Releaser pheromone leads to rapid response, however it is quickly degraded.
- Sex pheromones are involved in the process of reproduction by attracting the opposite sex in animals. Release of sex pheromones indicates the availability of the female for breeding.
- Pheromones are the substances that are released into the environment by an individual of a species that elicits a specific social response in members of the same species. They are the chemicals used by insects and other animals to communicate with each other.
- Pheromones are majorly used in cattle and pig breeding. They are sold as commercial preparations in the market based on their structural analogues. However, in some farms, nasal rings are used with substances containing pheromones.
- Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female.

10.9 KEY WORDS

- **Pheromones traps:** A pheromone trap is used to capture the insect pest using specific pheromones.
- **Sex pheromone:** The sex pheromone which is found in female rodent urine or vaginal discharge has been used successfully as an important substance for attracting males to traps or to stations with toxic bait or chemosterilants.
- **Aggregation pheromones:** Aggregation pheromones are used to gather together individuals of the same species both males as well as females in moderately large numbers.
- **Alarm pheromones:** Alarm pheromones can trigger flight or aggression in individuals of the same species. Alarm pheromones are also used to warn members of the same species about upcoming danger.
- **Releaser pheromones:** Releaser pheromones are pheromones that cause an alteration in the behaviour of the recipient. Releaser pheromone leads to rapid response, however it is quickly degraded.

- **Artificial insemination:** Artificial insemination refers to the process of collecting sperm cells from a genetically superior male and manually depositing these sperms into the reproductive tract of a similar, i.e., genetically superior female.

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10.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the pheromones.
2. State the pheromone trap.
3. Elaborate on the aggregate pheromones.
4. Interpret the artificial insemination.
5. Define the embryo transfer technology.

Long-Answer Questions

1. Discuss the role of pheromones in insect control.
2. Briefly define the role of pheromones in rodent control.
3. Describe the Pheromones. Write the classification of pheromones.
4. Write about the uses, advantages and disadvantages of using pheromones.
5. Analyse the role of pheromones in Animal breeding.

10.11 FURTHER READINGS

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BLOCK - IV
BIOTECHNOLOGY IN MEDICINE

NOTES

UNIT 11 STEM CELLS

Structure

11.0 INTRODUCTION

The term stem cell was coined by Theodor Boveri and Valentin Haecker in late 19th century. Pioneering works in theory of blood stem cell were conducted in the beginning of 20th century by Artur Pappenheim, Alexander Maximow, and Ernst Neumann. The key properties of a stem cell were first defined by Ernest McCulloch and James Till at the University of Toronto in the early 1960s. They discovered the blood-forming stem cell, the Hematopoietic Stem Cell (HSC), through their pioneering work in mice. McCulloch and Till began a series of experiments in which bone marrow cells were injected into irradiated mice.

Stem cells are undifferentiated or partially differentiated cells that can differentiate into various types of cells and proliferate indefinitely to produce more of the same stem cell. They are the earliest type of cell in a cell lineage. They are found in both embryonic and adult organisms, but they have slightly different properties in each. They are usually distinguished from progenitor cells, which cannot divide indefinitely, and precursor or blast cells, which are usually committed to differentiating into one cell type.

Adult stem cells are found in a few select locations in the body, known as niches, such as those in the bone marrow or gonads. They exist to replenish rapidly lost cell types and are multipotent or unipotent, meaning they only differentiate into a few cell types or one cell type. In mammals, they include, among others, hematopoietic stem cells, which replenish blood and immune cells, basal cells, which maintain the skin epithelium, and mesenchymal stem cells, which maintain bone, cartilage, muscle and fat cells. Adult stem cells are a small minority of cells;

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they are vastly outnumbered by the progenitor cells and terminally differentiated cells that they differentiate into.

In practice, stem cells are identified by whether they can regenerate tissue. For example, the defining test for bone marrow or Hematopoietic Stem Cells (HSCs) is the ability to transplant the cells and save an individual without HSCs.

In this unit, you will study about the embryonic, adult, hematopoietic, epithelial, and mesenchymal stem cells, application of stem cells, knock out, and knock in technology.

11.1 OBJECTIVES

After going through this unit, you will be able to:

- Explain the embryonic, adult, hematopoietic, epithelial, and mesenchymal stem cells
- Elaborate on the application of stem cells
- Analyse the knock out and knock in technology

11.2 STEM CELLS

Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body. As, we can see in the figure below, they can differentiate to produce different types of cell:-

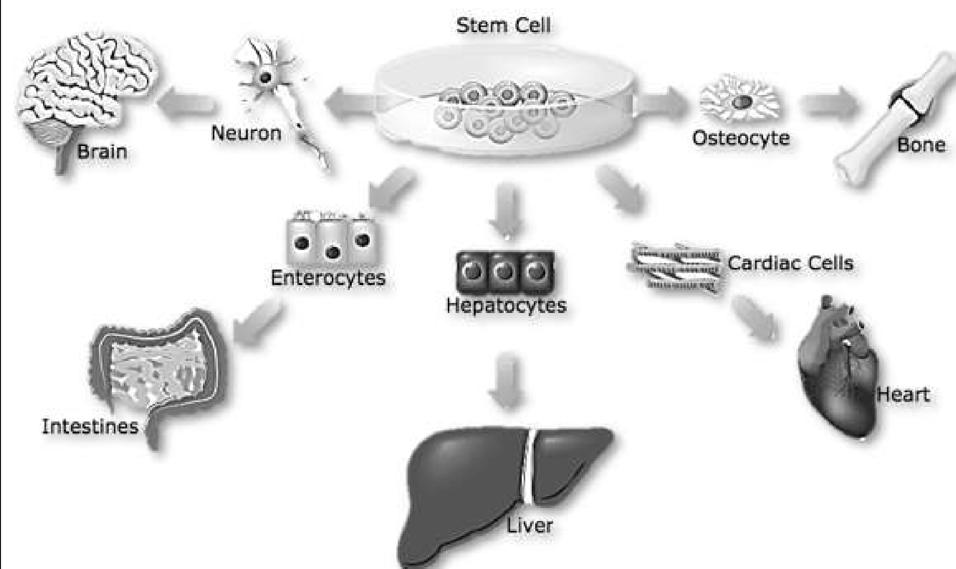


Fig. 11.1 *Differentiation of Stem Cells to Form Different Types of Cells*

Stem cells have unique properties:

1. They are unspecialized cells.
2. They can undergo self-replication via asymmetric cell division.

3. They can undergo differentiation to produce specific cells in accordance with the need of the body as well as signal received by the stem cells.

11.2.1 Types of Stem Cells

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Stem cells can be classified:

A. On the basis of their potency
B. On the basis of their origin

The picture below summarizes different types of stem cells on the basis of potency as well as origin:-

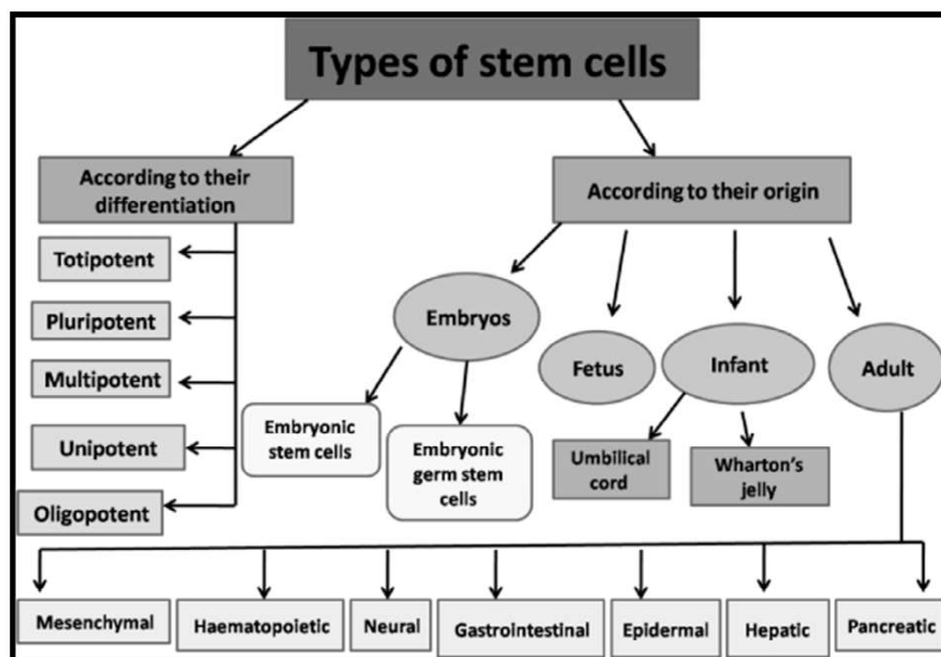


Fig. 11.2 Classification of Stem Cells

Let us discuss both the ways one by one:

A. On the basis of potency, i.e., their ability to differentiate and form different types of cells, stem cells are of following types:-
   1. Totipotent: These stem cells can differentiate into all possible cell types. Oocytes and sperm are the best differentiated cells in our body and they are capable of forming any tissue in the body.
   2. Pluripotent: These cells can turn into almost any cell. Cells derived from the early embryo, i.e., blastocyst are referred to as pluripotent.
   3. Multipotent: These cells are capable of differentiating into a closely related family of cells, i.e., these cells that can only give rise to cells of the tissue from which they are isolated. For example, Adult hematopoietic stem cells can differentiate to become red and white blood cells or platelets.

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4. Oligopotent: These cells can differentiate into a few different cell types. For example- Adult lymphoid or myeloid stem cells.
5. Unipotent: These cells are capable of producing only one kind of cell, i.e., their own cell type. Their self-renewal property helps in repair of the damaged adult tissue. For examples - adult muscle stem cells.

B. On the basis of their origin

1. Embryonic Stem Cells (ESCs)
   - They are derived from early stages of embryo development, i.e., from mouse or human blastocyst
   - They are pluripotent, self-renewing cells
   - They can be stored in culture for a long duration of time as undifferentiated cell lines
   - These undifferentiated cells can be stimulated to differentiate into any cell line
   - ESCs can differentiate into endoderm, mesoderm, and ectoderm embryonic germ layers or any type of somatic cells.
   - They are of wide significance in tissue regeneration therapy
   - Embryonic stem cells are also derived in-vitro as shown in the figure below:

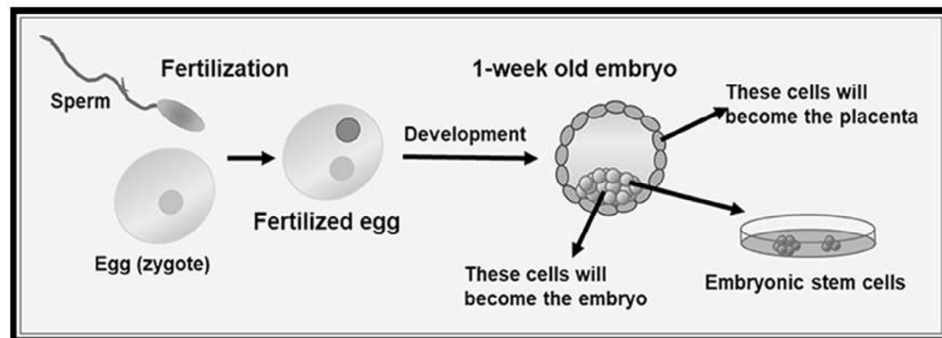


Fig. 11.3 *In-Vitro Isolation of Embryonic Stem Cells*

2. Embryonic Germ Stem Cells
   - They are derived from Primordial Germline Cells (PGCs) in the early development.
   - The PGC-derived cells are pluripotent.
3. Fetal Stem Cells
   - These cells are present in the organs of the fetuses.
   - They are capable of differentiating into two different types of stem cells, i.e., pluripotent stem cells and hematopoietic stem cells.

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4. Umbilical Cord Stem Cells
   - They are derived from the Umbilical cord
   - Stem cells derived from Umbilical cord blood varies when compared to stem cells derived from bone marrow and adult peripheral blood
   - Umbilical Cord cells are multipotent
   - They can differentiate into neurons and liver cells
   - Below, the figure depicts the methodology of collecting blood form umbilical cord:-

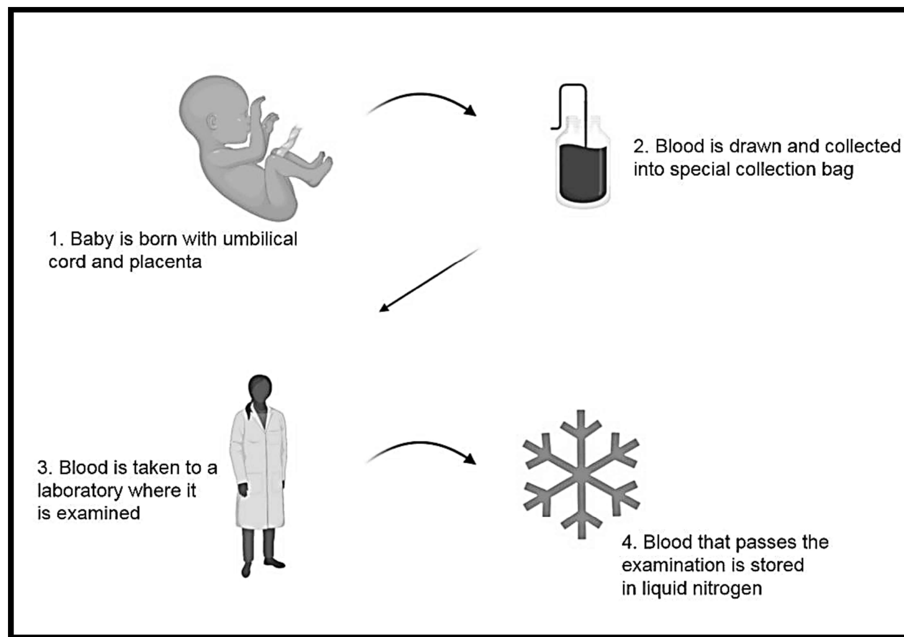


Fig. 11.4 *Collection of Blood from Umbilical Cord*

5. Wharton's Jelly
   - Wharton's jelly, refers to the umbilical cord matrix
   - It serves as a source of mesenchymal stem cells.
   - Wharton's jelly cells can be propagated for long duration
   - They can be induced to differentiate in vitro into neurons
6. Adult Stem Cell
   - Adult stem cells are the stem cells which are derived from mature tissue.
   - They can be isolated from the tissues of a fully developed child (whole embryo) or adult.
   - They are multipotent in nature.
   - They play a crucial role in the physiological processes like tissue repair, regeneration, etc.

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- Below we have mentioned, different types of adult stem cells:
  (i) **Mesenchymal Stem Cells:** Initially, Mesenchymal Stem Cells (MSCs) were described as adherent cells with a fibroblast-like appearance which can differentiate into several other cell types such as osteocytes, chondrocytes, adipocytes, tenocytes, and myocytes. MSCs can be derived from the connective tissue or stroma that surrounds the body's organs and other tissues. MSCs exhibits property of long-storage without major loss of their potency.
  (ii) **Hematopoietic Stem Cells:** Due to their self-renewal properties, Hematopoietic Stem Cells (HSCs) are capable to differentiate into different cells of all hematopoietic lineages. They can be used to treat hematologic disorders. The following figure depicts the mode of division of HSCs:

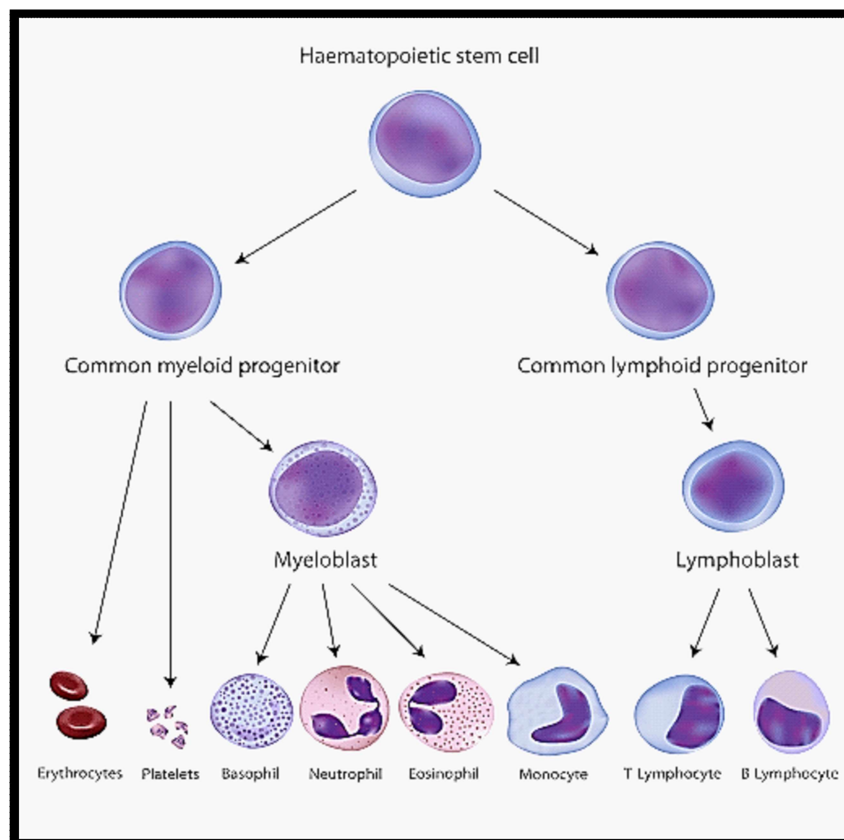


Fig. 11.5 *Lineage of Blood Cells*

  (iii) **Neural Stem Cells:** They are present in specialized molecular microenvironments in the adult mammalian brain. Due to their multipotency and self-renewal properties, they can be potentially used in cellular therapy of the brain.

(iv) **Gastrointestinal Stem Cells:** Gastrointestinal Stem Cells (GSCs) are located in the intestinal crypts and gastric glands. However, the position and mechanism of these stem cells is widely debated.

(v) **Epidermal Stem Cells:** Epidermis undergoes a lot of wear and tear and hence it is a rapidly rejuvenating tissue. Epidermal stem cells are established in the basal layer and due to their self-renewal properties participate significantly in maintaining homeostasis and cellular regeneration of normal skin; wound healing as well as neoplasm formation.

(vi) **Hepatic Stem Cells:** As we all know, hepatic (liver) tissue possesses a strong regenerative capacity and can utilizing diverse means of regeneration depending upon the kind and degree of the wound or injury. Mature liver cells or hepatic cells can propagate to replace the damaged tissue and hence let the revival of the parenchymal function.

As we have learned in the previous section that scientists have found stem cells in tissues, including:

- Bone marrow
- The brain
- Skeletal muscles
- Blood and blood vessels
- The liver
- Skin

These adult stem cells can divide or self-renew indefinitely. Thus, it can be concluded that an adult person's body contains stem cells throughout the life and the body can utilize these stem cells in accordance with the need and signal received. Adult stem cells remain in a non-specific state, till the body requires them for a definite purpose such as regeneration or maintenance of body tissues or repair. However, adult stem cells are difficult to be located and isolated. They can remain in a non-dividing and non-specific state for years until the body signals them to repair, regenerate or develop some new tissue.

11.2.2 Application of Stem Cells

Stem cells under optimum conditions and on receiving proper signal can regenerate damaged tissue which potentially could save several lives or repair wounds or can heal tissue damage in people after some serious illness or injury. Below, we have listed some potential use of stem cells:

1. **Tissue Regeneration**

The most common example for use of stem cell is tissue regeneration. Until now, if anyone needs a new kidney for replacing the damaged one, he or she had to wait

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for a donor before undergoing transplantation. However, if stem cells are available, one can instruct stem cells to differentiate in a certain way to produce a specific type of tissue or organ. For ex- surgeon uses stem cells from just beneath the skin's surface to make new skin tissue. These newly transplanted cells can easily repair or regenerate skin injury or other severe burn.

2. **Cardiovascular Disease Treatment**

In a study conducted in 2013, a team of researchers reported in "*PNAS Early Edition*" (a scientific journal) that they had created blood vessels in laboratory mice, using human stem cells. It was reported that a network of newly formed blood vessel were established after 2 weeks of transplanting the stem cells. Researchers believed that this technique could be developed to treat people having chronic vascular and cardiovascular diseases.

3. **Brain Disease Treatment**

Researchers are trying to differentiate embryonic stem cells into specific brain cells and tissues for treating specific brain disorders such as Parkinson's and Alzheimer's.

4. **Cell Deficiency Therapy**

Researchers are trying to develop healthy heart cells in a laboratory to treat people having serious cardiovascular disorders. These newly transplanted cells could easily repair heart damage. Similarly, people with type I diabetes could receive pancreatic cells to replace the insulin-producing cells that their own immune systems have lost or destroyed.

5. **Blood Disease Treatments**

Researchers use adult hematopoietic stem cells to treat serious disorders like sickle cell anaemia, leukaemia, and other immunodeficiency problems. Hematopoietic stem cells can undergo self-replication and differentiation to form all type of blood cells such as RBCs which carry oxygen and carbon dioxide in blood and WBCs that fight diseases.

6. **Research and Scientific Discovery**

Stem cells are widely used for research fields related to regeneration, repair mechanism, replication, differentiation, cell signalling, cell division, abnormal cell division leading to cancer as well as testing drugs on organ derived from stem cells rather than using them on human volunteers.

11.3 DONATING OR HARVESTING STEM CELLS

Stem cell can be derived from following sources:

1. **Bone Marrow**: These cells are usually taken from the hip or pelvic bone. Technicians then isolate the stem cells from the bone marrow for donation or storage.

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2. **Peripheral Stem Cells**: The donor receives numerous injections that stimulate their bone marrow to liberate stem cells into the blood. Subsequently, blood is taken out from the body, stem cells are separated from other blood cells via a machine and finally the filtered blood (blood without stem cells) is returned to the body.
3. **Umbilical Cord Blood**: This is a harmless and painless technique in which stem cells can be easily harvested from umbilical cord after delivery. Some people donate the cord blood cells whereas others store it for future purpose. The advantages of storing stem cells for future needs include:
   - Easily available when required in urgency like serious illness.
   - Very low chances of transplanted tissue being rejected when stem cells comes from the recipient's own body.

11.4 KNOCK IN AND KNOCK OUT TECHNOLOGY

Knock-in and knock-out technologies are quite dissimilar. These technologies and the methods are used to delete or inactivate a portion of the DNA sequences. Knock-ins produce a one-for-one substitution of the DNA sequence within the genome. Especially, knock-in technology is deals with the specific locus of the sequence, compared to how knock-out mouse generating technologies and methods focus on engineering an entirely new DNA sequence.

Knock - In Technology

A gene knock-in (abbreviation: KI) refers to a genetic engineering method that involves the one-for-one substitution of DNA sequence information in a genetic locus or the insertion of sequence information not found within the locus. Typically, this is done in mice since the technology for this process is more refined and there is a high degree of shared sequence complexity between mice and humans. The difference between knock-in technology and traditional transgenic techniques is that a knock-in involves a gene inserted into a specific locus, and is thus a "Targeted" insertion. It is the opposite of gene knock-out.

A common use of knock-in technology is for the creation of disease models. It is a technique by which scientific investigators may study the function of the regulatory machinery (e.g. promoters) that governs the expression of the natural gene being replaced. This is accomplished by observing the new phenotype of the organism in question. The BACs and YACs are used in this case so that large fragments can be transferred.

Knock-in technology is different from knock-out technology in that knock-out technology aims to either delete part of the DNA sequence or insert irrelevant DNA sequence information to disrupt the expression of a specific genetic locus. Gene knock-in technology, on the other hand, alters the genetic locus of interest

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via a one-for-one substitution of DNA sequence information or by the addition of sequence information that is not found on said genetic locus. A gene knock-in therefore can be seen as a gain of function mutation and a gene knock-out a loss of function mutation, but a gene knock-in may also involve the substitution of a functional gene locus for a mutant phenotype that results in some loss of function.

Gene knock-in originated as a slight modification of the original knock-out technique developed by Martin Evans, Oliver Smithies, and Mario Capecchi. Traditionally, knock-in techniques have relied on homologous recombination to drive targeted gene replacement, although other methods using a transposon-mediated system to insert the target gene have been developed. The use of loxP flanking sites that become excised upon expression of crerecombinase with gene vectors is an example of this. Embryonic stem cells with the modification of interest are then implanted into a viable blastocyst, which will grow into a mature chimeric mouse with some cells having the original blastocyst cell genetic information and other cells having the modifications introduced to the embryonic stem cells. Subsequent offspring of the chimeric mouse will then have the gene knock-in.

Gene knock-in has allowed, for the first time, hypothesis-driven studies on gene modifications and resultant phenotypes. Mutations in the human p53 gene, for example, can be induced by exposure to Benzo(a)Pyrene (BaP) and the mutated copy of the p53 gene can be inserted into mouse genomes. Lung tumours observed in the knock-in mice offer support for the hypothesis of BaP's carcinogenicity. More recent developments in knock-in technique have allowed for pigs to have a gene for green fluorescent protein inserted with a CRISPR/Cas9 system, which allows for much more accurate and successful gene insertions. The speed of CRISPR/Cas9-mediated gene knock-in also allows for biallelic modifications to some genes to be generated and the phenotype in mice observed in a single generation, an unprecedented timeframe.

Because of the success of gene knock-in methods thus far, many clinical applications can be envisioned. Knock-in of sections of the human immunoglobulin gene into mice has already been shown to allow them to produce humanized antibodies that are therapeutically useful. It should be possible to modify stem cells in humans to restore targeted gene function in certain tissues, for example, possibly correcting the mutant Gamma-chain gene of the IL-2 receptor in hematopoietic stem cells to restore lymphocyte development in people with X-linked severe combined immunodeficiency.

While gene knock-in technology has proven to be a powerful technique for the generation of models of human disease and insight into proteins in vivo, numerous limitations still exist. Many of these are shared with the limitations of knock-out technology. First, combinations of knock-in genes lead to growing complexity in the interactions that inserted genes and their products have with other sections of the genome and can therefore lead to more side effects and difficult-to-explain phenotypes. Also, only a few loci, such as the ROSA26 locus have been

characterized well enough where they can be used for conditional gene knock-ins; making combinations of reporter and transgenes in the same locus problematic.

The biggest disadvantage of using gene knock-in for human disease model generation is that mouse physiology is not identical to that of humans and human orthologs of proteins expressed in mice will often not wholly reflect the role of a gene in human pathology. This can be seen in mice produced with the ÄF508 fibrosis mutation in the CFTR gene, which accounts for more than 70% of the mutations in this gene for the human population and leads to cystic fibrosis. While ÄF508 CF mice do exhibit the processing defects characteristic of the human mutation, they do not display the pulmonary pathophysiological changes seen in humans and carry virtually no lung phenotype. Such problems could be ameliorated by the use of a variety of animal models, and pig models (pig lungs share many biochemical and physiological similarities with human lungs) have been generated in an attempt to better explain the activity of the ÄF508 mutation.

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Knock - Out Technology

A gene knock-out (abbreviation: KO) is a genetic technique in which one of an organism's genes is made inoperative ("Knocked Out" of the organism). However, KO can also refer to the gene that is knocked out or the organism that carries the gene knock-out. Knock-out organisms or simply knock-out s are used to study gene function, usually by investigating the effect of gene loss. Researchers draw inferences from the difference between the knock-out organism and normal individuals.

The KO technique is essentially the opposite of a gene knock-in technology. Knocking out two genes simultaneously in an organism is known as a Double Knock-Out (DKO). Similarly the terms Triple Knock - Out (TKO) and Quadruple Knock-Outs (QKO) are used to describe three or four knocked out genes, respectively. However, one needs to distinguish between heterozygous and homozygous KOs. In the former, only one of two gene copies (alleles) is knocked out, in the latter both are knocked out.

Knock-out s are accomplished through a variety of techniques. Originally, naturally occurring mutations were identified and then gene loss or inactivation had to be established by DNA sequencing or other methods.

1. **Homologous Recombination**

Traditionally, homologous recombination was the main method for causing a gene knock-out. This method involves creating a DNA construct containing the desired mutation. For knock-out purposes, this typically involves a drug resistance marker in place of the desired knock-out gene. The construct will also contain a minimum of 2kb of homology to the target sequence. The construct can be delivered to stem cells either through microinjection or electroporation. This method then relies on the cell's own repair mechanisms to recombine the DNA construct into the existing DNA. This results in the sequence of the gene being altered, and most

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cases the gene will be translated into a non-functional protein, if it is translated at all. However, this is an inefficient process, as homologous recombination accounts for only 10"2 to 10-3 of DNA integrations. Often, the drug selection marker on the construct is used to select for cells in which the recombination event has occurred.

2. **Site-Specific Nucleases**

There are currently three methods in use that involve precisely targeting a DNA sequence in order to introduce a double-stranded break. Once this occurs, the cell's repair mechanisms will attempt to repair this double stranded break, often through Non-Homologous End Joining (NHEJ), which involves directly ligating the two cut ends together. This may be done imperfectly, therefore sometimes causing insertions or deletions of base pairs, which cause frame shift mutations. These mutations can render the gene in which they occur non-functional, thus creating a knockout of that gene. This process is more efficient than homologous recombination, and therefore can be more easily used to create biallelic knockouts.

3. **Zinc-Fingers**

Zinc-finger nucleases consist of DNA binding domains that can precisely target a DNA sequence. Each zinc finger can recognize codons of a desired DNA sequence, and therefore can be modularly assembled to bind to a particular sequence. These binding domains are coupled with a restriction endonuclease that can cause a Double Stranded Break (DSB) in the DNA. Repair processes may introduce mutations that destroy functionality of the gene.

4. **TALENS**

Transcription Activator-Like Effector Nucleases (TALENs) also contain a DNA binding domain and a nuclease that can cleave DNA. The DNA binding region consists of amino acid repeats that each recognize a single base pair of the desired targeted DNA sequence. If this cleavage is targeted to a gene coding region, and NHEJ-mediated repair introduces insertions and deletions, a frame shift mutation often results, thus disrupting function of the gene.

5. **CRISPR/Cas9**

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 is a method for genome editing that contains a guide RNA complexes with a Cas9 protein. The guide RNA can be engineered to match a desired DNA sequence through simple complementary base pairing, as opposed to the time-consuming assembly of constructs required by zinc-fingers or TALENs. The coupled Cas9 will cause a double stranded break in the DNA. Following the same principle as zinc-fingers and TALENs, the attempts to repair these double stranded breaks often result in frame shift mutations that result in a non-functional gene.

A conditional gene knockout allows gene deletion in a tissue in a time specific manner. This is required in place of a gene knockout if the null mutation would lead to embryonic death. This is done by introducing short sequences called loxP sites around the gene. These sequences will be introduced into the germ-line via

the same mechanism as a knock-out. This germ-line can then be crossed to another germline containing crerecombinase which is a viral enzyme that can recognize these sequences, recombines them and deletes the gene flanked by these sites.

Knockouts are primarily used to understand the role of a specific gene or DNA region by comparing the knockout organism to a wild type with a similar genetic background. Knockout organisms are also used as screening tools in the development of drugs, to target specific biological processes or deficiencies by using a specific knockout, or to understand the mechanism of action of a drug by using a library of knockout organisms spanning the entire genome, such as in Saccharomyces Cerevisiae.

Check Your Progress

1. Explain the stem cells.
2. Write down the properties of stem cells.
3. Differentiate between the multipotent and unipotent cells.
4. What are epidermal stem cells?
5. Elaborate on the adult stem cells.
6. Define the knock- in technology.
7. Illustrate the knock- out technology.

11.5 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
2. Stem cells have unique properties:-
   - o They are unspecialized cells.
   - o They can undergo self-replication via asymmetric cell division.
   - o They can undergo differentiation to produce specific cells in accordance with the need of the body as well as signal received by the stem cells.
3. Multipotent cells are capable of differentiating into a closely related family of cells, i.e., these cells that can only give rise to cells of the tissue from which they are isolated, while unipotent cells are capable of producing only one kind of cell, i.e., their own cell type. Their self-renewal property helps in repair of the damaged adult tissue.
4. Epidermal stem cells are established in the basal layer and due to their self-renewal properties participate significantly in maintaining homeostasis and cellular regeneration of normal skin; wound healing as well as neoplasm formation.

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5. Adult stem cells are the stem cells which are derived from mature tissue. They can be isolated from the tissues of a fully developed child (whole embryo) or adult.
6. A gene knock-in (abbreviation: KI) refers to a genetic engineering method that involves the one-for-one substitution of DNA sequence information in a genetic locus or the insertion of sequence information not found within the locus.
7. A gene knock-out (abbreviation: KO) is a genetic technique in which one of an organism's genes is made inoperative ("Knocked Out" of the organism). However, KO can also refer to the gene that is knocked out or the organism that carries the gene knock-out.

11.6 SUMMARY

- Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
- Totipotent: These stem cells can differentiate into all possible cell types. Oocytes and sperm are the best differentiated cells in our body and they are capable of forming any tissue in the body.
- Multipotent: These cells are capable of differentiating into a closely related family of cells, i.e., these cells that can only give rise to cells of the tissue from which they are isolated.
- Unipotent: These cells are capable of producing only one kind of cell, i.e., their own cell type. Their self-renewal property helps in repair of the damaged adult tissue. For examples - adult muscle stem cells.
- Mesenchymal Stem Cells (MSCs) were described as adherent cells with a fibroblast-like appearance which can differentiate into several other cell types such as osteocytes, chondrocytes, adipocytes, tenocytes, and myocytes. MSCs can be derived from the connective tissue or stroma that surrounds the body's organs and other tissues.
- Gastrointestinal Stem Cells (GSCs) are located in the intestinal crypts and gastric glands. However, the position and mechanism of these stem cells is widely debated.
- Stem cells under optimum conditions and on receiving proper signal can regenerate damaged tissue which potentially could save several lives or repair wounds or can heal tissue damage in people after some serious illness or injury.
- The most common example for use of stem cell is tissue regeneration. Until now, if anyone needs a new kidney for replacing the damaged one, he or she had to wait for a donor before undergoing transplantation.

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- Knock-in and knock-out technologies are quite dissimilar. These technologies and the methods are used to delete or inactivate a portion of the DNA sequences. Knock-ins produce a one-for-one substitution of the DNA sequence within the genome.
- A gene knock-in (abbreviation: KI) refers to a genetic engineering method that involves the one-for-one substitution of DNA sequence information in a genetic locus or the insertion of sequence information not found within the locus.
- A gene knock-out (abbreviation: KO) is a genetic technique in which one of an organism's genes is made inoperative ("Knocked Out" of the organism). However, KO can also refer to the gene that is knocked out or the organism that carries the gene knock-out.

11.7 KEY WORDS

- **Stem cells:** Stem cells are defined as undifferentiated cells that can divide and differentiate into specific cells, in accordance with the need of the body.
- **Totipotent:** These stem cells can differentiate into all possible cell types. Oocytes and sperm are the best differentiated cells in our body and they are capable of forming any tissue in the body.
- **Pluripotent:** These cells can turn into almost any cell. Cells derived from the early embryo, i.e., blastocyst are referred to as pluripotent.
- **Oligopotent:** These cells can differentiate into a few different cell types. For example- Adult lymphoid or myeloid stem cells.
- **Unipotent:** These cells are capable of producing only one kind of cell, i.e., their own cell type.
- **Knock - in technology:** A gene knock-in (abbreviation: KI) refers to a genetic engineering method that involves the one-for-one substitution of DNA sequence information in a genetic locus or the insertion of sequence information not found within the locus.
- **Knock - out technology:** A gene knock-out (abbreviation: KO) is a genetic technique in which one of an organism's genes is made inoperative ("Knocked Out" of the organism).

11.8 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Define the stem cells.
2. State the properties of stem cells.

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3. Differentiate between multipotent and unipotent cells.
4. Explain the epidermal stem cells.
5. Illustrate the adult stem cells.
6. Elaborate on the knock- in technology.
7. Define the knock- out technology.

Long-Answer Questions

1. Describe the stem cells with its different types, and applications.
2. Explain the properties of stem cells with the help of examples.
3. Define the steps of isolating umbilical cord cells.
4. Briefly discuss the different types of adult stem cells.
5. Analyse the knock –in and knock-out technology.

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UNIT 12 RECOMBINANT VACCINES

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Structure

12.0 INTRODUCTION

A vaccine produced through recombinant DNA technology, is called recombinant vaccine. By inserting the DNA encoded antigen (such as a bacterial surface protein) into the bacterial or mammalian cells, immune system can be stimulates. After expressing the antigen in these cells, purifying it from them.

Recombinant vaccines are made by using bacterial or yeast cells. A small piece of DNA is taken from the virus or bacterium against which, we want to protect and inserted into the manufacturing cells. By combining the physiology of one micro-organism and the DNA of another, immunity can be created against diseases that have complex infection processes. An example is the RVSV-ZEBOV vaccine licensed to Merck that is being used in 2018 to combat Ebola in Congo.

The terms vaccine and vaccination are derived from Variolae vaccinae (smallpox of the cow), the term devised by Edward Jenner (who both developed the concept of vaccines and created the first vaccine) to denote cowpox. He used the phrase in 1798 for the long title of his "*Inquiry into the Variolae Vaccinae Known as the Cow Pox*", in which he described the protective effect of cowpox

against smallpox. In 1881, to honour Jenner, Louis Pasteur proposed that the terms should be extended to cover the new protective inoculations then being developed. The science of vaccine development and production is termed vaccinology.

A subunit vaccine is a vaccine that presents one or more antigens to the immune system without introducing pathogen particles, whole or otherwise. The word "Subunit" simply means the antigen is a fragment of the pathogen, and the antigens involved can be any molecule, such as proteins, peptides or polysaccharides. Just like inactivated vaccines, the vaccine is completely "Dead", and is therefore less risky.

In this unit, you will study about the recombinant vaccines, subunit vaccines, live vaccines, production of insulin, and tissue plasminogen activator.

12.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the recombinant vaccines
- Explain the subunit vaccines
- Elaborate on the live vaccines
- Define the production of insulin
- Interpret the tissue plasminogen activator

12.2 RECOMBINANT VACCINES

Recombinant DNA technology refers to the joining together of genetic material, i.e., DNA molecules from two different species. The recombined DNA molecule is then inserted into a host organism to produce new genetic combinations that are of significant value to fields like medicine, science, agriculture or industry. The steps involved in the entire process of recombinant DNA technology can be summarized as follows:-

1. Identification of the Desirable Gene

The first step is the identification of the desirable gene or gene of interest that codes for a specific target protein. A gene that codes for a desirable trait or protein must first be identified. Several molecular techniques like Gene Chips (Microarray) and DNA sequencing can be used to identify the desirable gene.

2. Isolation of Desirable Gene

The second step involves the isolation of the desirable gene from the target species. It can be achieved either via mechanically breaking the cells or with the

aid of chemical agents like detergents. The entire DNA can be then separated from the other cell components via technique known as cell centrifugation. Now, to separate the target gene from the total DNA content following steps needs to be followed:

- Separation of DNA fragments according to size via Gel Electrophoresis.
- Identification of the gene of interest using a DNA probe.
- Cut out of the gel and amplified (copied) using PCR.
- Alternatively, gene of interest could be inserted into a bacterial plasmid using the enzyme DNA Ligase.
- Bacteria would automatically copy the gene while undergoing cell division- a technique popularly called as Gene Cloning.
- However, if enough information is available regarding the gene of interest then it might be possible to create specific DNA primers and copy the gene using PCR without isolating it on a gel.

3. Transformation of the Desirable Gene

- Finally, a vector (varies according to cell type) is used to transfer the gene of interest into the organism being modified.
- The final DNA sequence that is prepared consisting of target gene and associated regulatory sequences (promoter and termination) sequences is referred to as Gene Construct.
- However, the success rate at which transgene is expressed is very low.
- For the target gene to be expressed, it must make its way into the nucleus.
- For it to be passed on during cell division (mitosis and meiosis) it must integrate itself into the target cells genome via recombination /crossing over.
- For verifying, whether, the target gene has been inserted into the genome or not- researchers incorporate a second gene known as reporter gene into the gene construct. This second gene codes for an easily selectable/ observable characteristic like antibiotic resistance or glow in the dark protein.
- This enables the researchers to easily verify whether the integrated gene is expressing or not.

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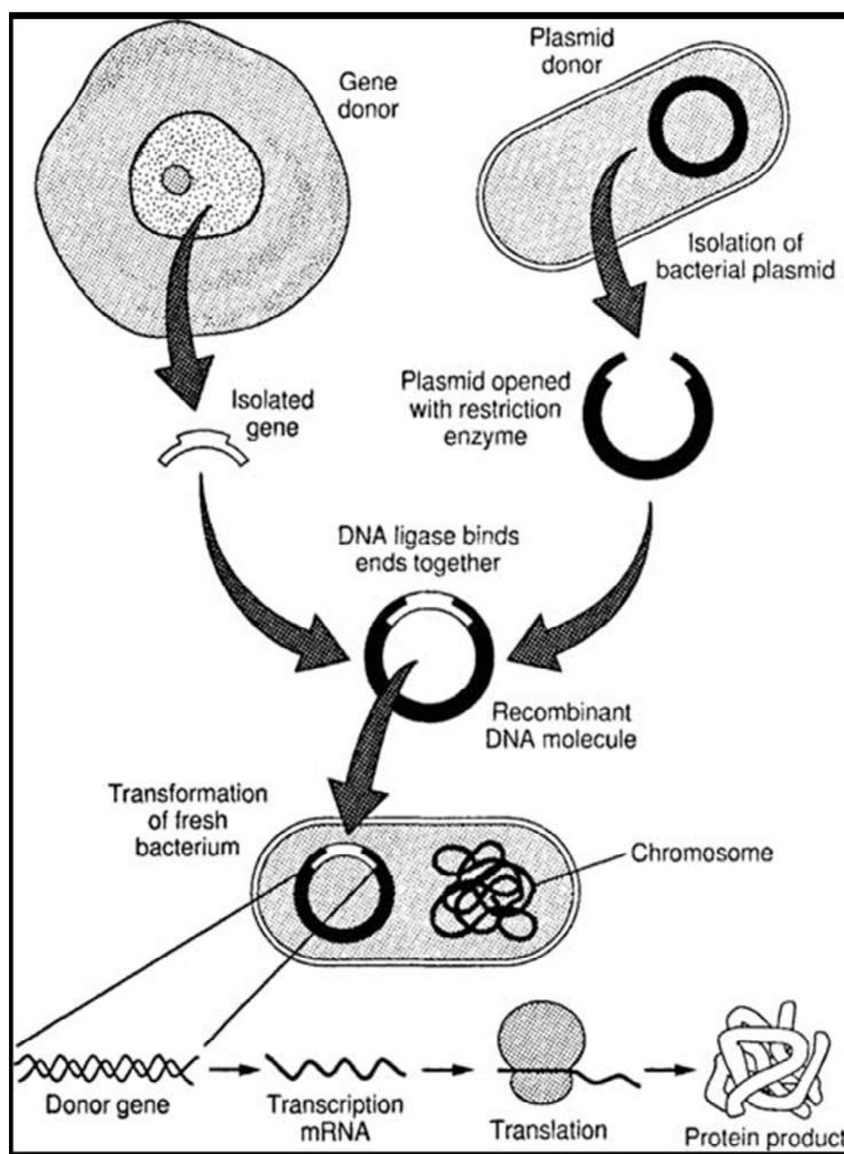


Fig. 12.1 *Steps Involved in the Process of Recombinant DNA Technology*

However, genetically engineered host cells for production of recombinant proteins must be grown in large quantities. To achieve this, 'Bioreactors' are used for making pharmaceutical products such as antibiotics and insulin in large quantities. A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity.

The three main stages to a bio-process consist of: - upstream processing, bio-reaction and downstream processing. In the next section, we will discuss each stage in detail:

A. Upstream Processing

In this step, raw material isolated from a biological or non-biological origin is transformed to a more suitable form for processing.

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B. Bioreaction

Next stage, i.e., the bioreaction stage consists of three operations namely:-

(i) Production of biomass

(ii) Metabolise biosynthesis

(iii) Biotransformation

C. Downstream Processing

This is the final stage where the material is further transformed to a more useful form. This stage involves the basic steps of separating the cells from culture or broth, increasing the concentration of the recombinant protein, purifying the recombinant product and finally assessing the quality of the product.

Types of Bioreactor Processes

There are three main types of bioreactor processes which are used these are, batch, continuous, and fed-batch.

i. ***Batch Bioreactor*** **Processes:** Batch bioreactor progressions involve filling the bioreactor with substantial amount of medium as well as the inoculums and working the bioreactor with no additional nutrients or medium until after the growth profile is complete.

ii. ***Continuous Bioreactor Processes*****:** Continuous bioreactor progressions involve the bioreactor being constantly fed with required nutrients as well as the medium, as the reactor is persistently harvesting material. The harvested material is being collected continually resulting in larger volumes of harvested material and longer bioreactor campaigns. However, the only disadvantage associated with such long processes is that it drastically increases the chances of contamination.

iii. ***Fed-Batch Bioreactor Processes*****:** Fed-batch bioreactor progressions are the most common reactor processes used in the commercial setup. This methodology begins with a low starting volume and feeds nutrients as well as medium on a planned schedule without removing the harvest material. The entire harvest material is collected after the process is complete and then processed for downstream processing.

Scale-up process involves the progression of culture systems from small scale laboratory production to large scale industrial or commercial production. This methodology aims at increasing the scale of a culture depending upon the

proliferation of cells. All the scale up process can be broadly divided into two categories namely:

1. Scale-up in suspension-Scale-up of suspension culture chiefly involves an increase in the volume of the culture.
2. Scale-up in monolayer- The monolayer cultures are anchorage-dependent culture. This process involves increase in the surface area of the substrate in proportion to the number of cells as well as volume of the medium.

There are many different types of bioreactors like: stirred-tank, rocker, air lift and fixed-bed etc. which are designed specifically to scale up the process. In the next section, we will discuss different types of bioreactors used for scaling up process one by one:

(i) Stirred-Tank Bioreactors: Stirred-Tank Reactors (STRs) are the most widely-used bioreactors and are well equipped with an impeller for homogenizing the culture media as well as a sparger for delivering the oxygen to the cells. STRs vary in size from 15 mL to 2000 L for single-use in stainless-steel body. STRs are chiefly used to scale-up a procedure from research and development scale or laboratory scale to manufacturing or industrial scale. The major aim is to ensure that a process at a smaller volume can act as a representative of larger volumes.

(ii) Rocker Bioreactors: Rocker bioreactors are offered as single-use systems. They comprises of a bag on a moving platform, relying on a rocking motion for the mixing. Oxygen diffuses takes place through the headspace via liquid-gas interface, as rocket bioreactors lacks sparger which is used for delivering oxygen to the cells. Rocket bioreactors vary in size from a few litres to a maximum of 100 L. They are primarily used for small-scale production or for seeding into larger bioreactors.

(iii) Air Lift Bioreactors: Air lift bioreactors are not very often used in the biopharmaceuticals industry as they are not very popular with regulatory processes that largely remained unexplored. Air lift bioreactors rely on air bubbles to aerate as well as to carry the media around the reactor for mixing at the same time.

(iv) Fixed-Bed Bioreactors: Fixed-bed bioreactors are used for adherent-cells, i.e., the cell types that can only grow only when they are attached to a surface. Fixed-bed bioreactors usually have strips of fibres (carriers) which are packed together to make the fixed-bed. These carriers ensure a high surface area for the cells to adhere to. Aerated culture medium flows through it. Further, anchorage dependent cells can also be cultivated in other

bioreactors such as stirred-tank, rocker and air lift bioreactors just like the suspended cells. However, they require to be attached to 100-micrometre beads (i.e. micro-carriers) placed in suspension. Scale-up of this process is a tedious, laborious and time-consuming process when compared to suspension process or the adherent cells process in the desirable fixed-bed bioreactors.

12.3 TYPES OF RECOMBINANT VACCINES

In recent years, recombinant DNA technology has become a boon to produce new generation vaccines or novel recombinant vaccines. The recombinant vaccines may be broadly categorized into three categories namely: subunit vaccines, attenuated recombinant vaccines and vector recombinant vaccines. In this section, we will be discussing different types of recombinant vaccines:

12.3.1 Subunit Recombinant Vaccines

These vaccines are the essential components of the pathogenic organisms. Subunit vaccines include components of pathogenic organism like peptides, proteins, and DNA. The biggest advantage of using subunit recombinant vaccines is that they are pure, stable as well as safe to use. However, subunit vaccines are very expensive as well as have the possibility of alteration in native conformation of the pathogen's subunit used for producing vaccines. For instance: - Hepatitis B mainly affects liver causing chronic hepatitis, cirrhosis and sometimes it can cause severe liver cancer too. The core of the Hepatitis B virus contains a viral genome (DNA) bounded by a phospholipid envelope having surface antigens. However, it is not easy to grow hepatitis B virus in mammalian cell culture and produce surface antigens.

Recombinant hepatitis B vaccine is a subunit vaccine which is produced by cloning HbsAg gene, i.e., gene encoding for hepatitis B surface antigen in yeast cells (*Saccharomyces Cerevisiae*). The gene for HBsAg is inserted and these plasmids are then transferred and cultured in in tryptophan, free medium. The expression of the HBsAg gene leads to the production of 2nm sized particles which are similar to those seen in patients infected with hepatitis B virus. These particles can be isolated to immunize people against deadly hepatitis B virus.

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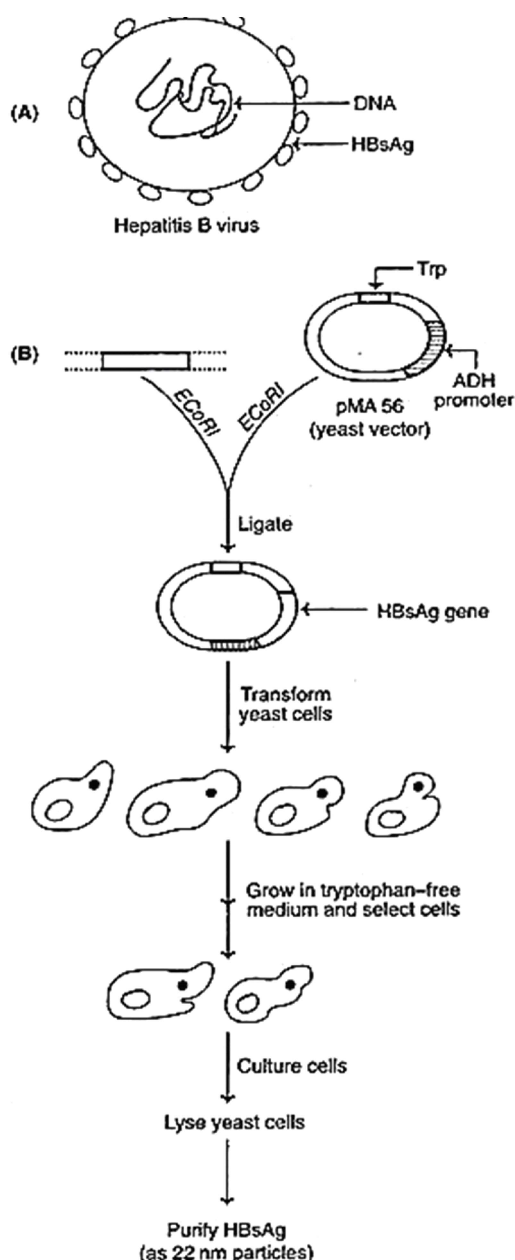


Fig. 12.2 *Production of Hepatitis B Virus Surface Antigen Particle in Yeast Cells*

12.3.2 Attenuated Recombinant Vaccines

In these types of vaccines, pathogenic organisms like viruses, bacteria etc. are genetically modified to make them non-pathogenic for producing recombinant vaccines. Earlier, attenuated strains of some highly pathogenic organisms were prepared by continued cultivation for several weeks, months or years. Such attenuated pathogens would lose its capability to cause disease however it retains the ability to act as an immunizing agent. Now a days, it is possible to genetically alter bacteria or viruses for generating live attenuated recombinant vaccines.

The genetic alteration involves either deletion of virulent genes of pathogens or manipulation of non-pathogenic organisms to express antigenic determinants from pathogenic organisms. The biggest advantage of using attenuated vaccines is that the original or native conformation of the antigenic determinants is preserved leading to highly effective immune response. For instance: - Intestinal disorder, Cholera, caused by Vibrio cholera is characterized by fever, abdominal pain or dehydration.

Current vaccines available for preventing Cholera immunize people maximum for a period of 6 months. Thus, researchers are trying to develop more effective vaccines against the deadly Cholera. By genetic engineering, it has become possible to permanently delete the DNA sequence encoding A1 peptide (functional component of the enterotoxin which is responsible for its toxic activity) leading to the formation of a non-pathogenic strain of V. cholera as it cannot produce enterotoxin. This genetically altered V. cholera is an excellent candidate to be used as an attenuated vaccine.

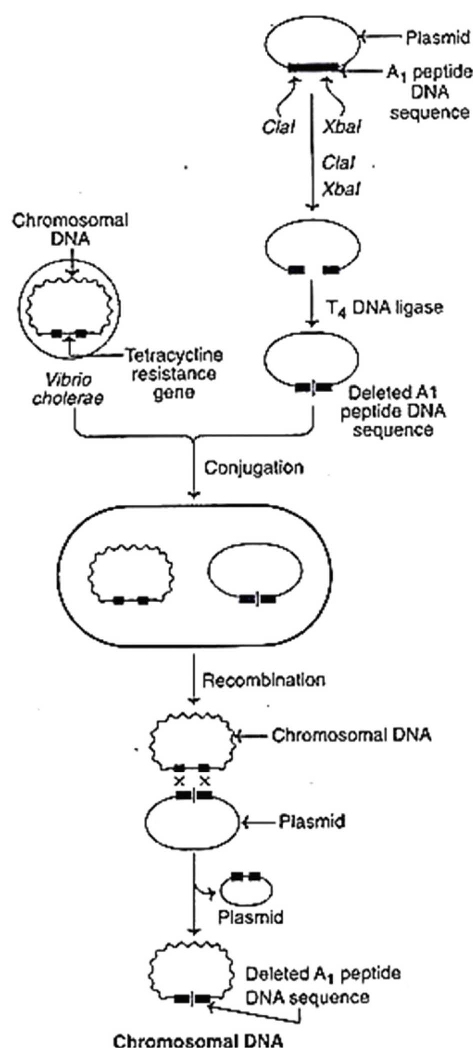


Fig. 12.3 *Development of New Strain Pf V. Cholerae as an Example of Attenuated Recombinant Vaccine*

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12.3.3 Vector Recombinant Vaccines

These vaccines are genetically modified viral vectors that can be used against certain pathogenic organism. For instance: - Vaccinia viruses is initially used by Jenner for the eradication of smallpox. This virus contains a double-stranded DNA of 187 kb that is responsible for encoding around 200 different proteins and can easily replicate in the host cell cytoplasm. The cloned foreign genes from a highly pathogenic organism can be inserted into vaccinia virus genome for encoding antigens which in turn produces antibodies against the disease-causing organism.

The biggest advantage of using vaccinia virus for making recombinant vaccines are that they are harmless, stable, easy to use as well as can stimulate both branches of immunity, i.e., humoral and cell mediated immunity. Such recombinant vector vaccines have been developed successfully against fatal diseases such as herpes simplex virus, influenza, hepatitis, rabies, etc. However, these vaccines are yet to be applied on humans due to safety concerns. The production of recombinant vaccinia virus is carried out by a two-step procedure as shown in the figure:

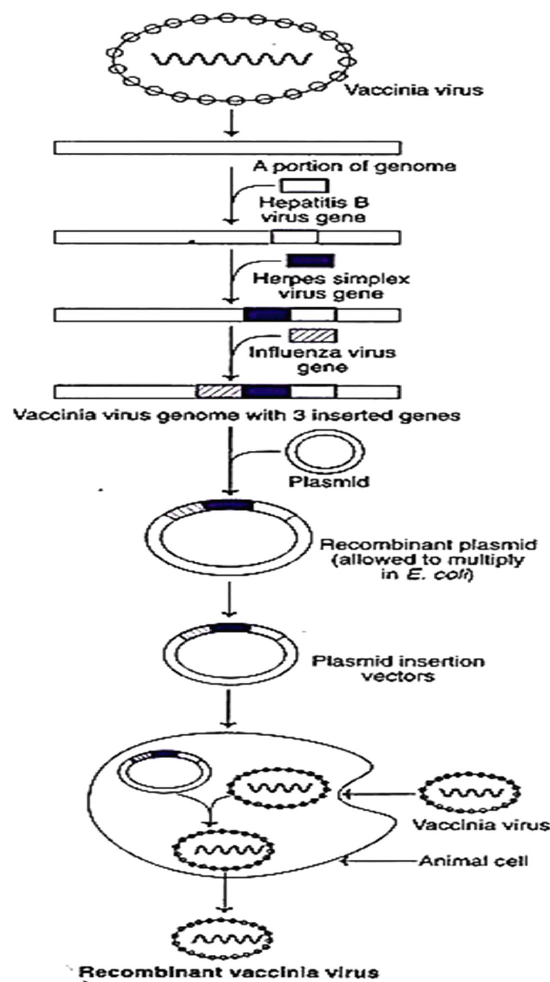


Fig. 12.4 *Production of Recombinant Vaccinia Virus*

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12.4 PRODUCTION OF PHARMACEUTICALLY ESSENTIAL COMPOUNDS

The most important application of recombinant DNA technology is the production of essential biomolecules like insulin, tissue plasminogen activators, blood products like clotting factor VIII, albumins, cytokines, follicle stimulating hormones, and interferon, etc. These artificially produced pharmaceutical products can be utilized effectively for improving the lifestyle of patients having lifestyle or genetic disorders. In the next section, we will be discussing the production of two biomolecules, i.e., insulin and tissue plasminogen activator by recombinant DNA technology in detail.

12.5 PRODUCTION OF RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator is the first pharmaceutical product produced using mammalian cell culture at Genentech in 1982. Tissue plasminogen activator is a protease enzyme which helps in dissolving blood clots. Under normal condition, plasmin degrades fibrin leading to dissolvation of blood clots. Tissue plasminogen activator has got very high therapeutic value and protects heart and brain from blood clots.

The host cells used in production of Tissue Plasminogen Activator (tPA) are as follows:

(i) Mammalian cells- They can be of two types:

   a. Non recombinant producers- They can be derived from extracts of human tissues such as uterus, adrenal, pituitary, kidney, muscle, heart, brain, liver, and testis, etc.

   b. Recombinant producers- Mammalian cells inserted with human tPA gene have been excellent producer of tPA when compared to other cell lines. For instance- cell type of Human melanoma, Bowes melanoma, etc.

(ii) Bacteria – Bacterium E.coli is one of the most suitable choices for production of tPA

(iii) Yeast and fungi- *Saccharomyces Cereviase* has been used for production of tPA but the yield has been too low. Similarly, production of tPA from *Aspergillus Nidulans* has been reported.

12.5.1 Steps Involved in the Production of Recombinant Tissue Plasminogen Activator

1. Recombinant DNA technology is widely used in the production of cDNA molecule complementary to the mature mRNA of gene encoding the essential tissue plasminogen activator.

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2. This cDNA is cloned into a synthetic plasmid by the detailed process explained in the previous section and introduced into mammalian cells.
3. After this, transformed mammalian cells are cultured in a suitable animal cell culture media.
4. This laboratory process is replicated at industrial scale leading to mass production of Tissue plasminogen activator. The science behind the mass production of biologically significant molecules is known as fermentation. For this, tissue plasminogen activator producing recombinant cells are transferred into fermenters to produce them in large quantity.
5. Finally, tissue plasminogen activator secreted into the culture media is isolated and purified. This process involves the basic steps of separating the cells from culture or broth, increasing the concentration of the recombinant protein, purifying the recombinant product and finally assessing the quality of the product as explained in the introductory section.
6. AITEPLASE and RETEPLASE are the examples of tissue plasminogen activator produced by recombinant DNA technology.

12.5.2 Uses of Recombinant Tissue Plasminogen Activator

- Tissue plasminogen activator helps in dissolving blood clots.
- It degrades the blood clot without reducing the blood clotting capability in other parts of the body.
- Tissue plasminogen activator can be easily administered intravenously.
- It acts rapidly when compared to other thrombolytic agents.
- Finally, it has least side effects when compared to other thrombolytic agents.

12.6 PRODUCTION OF RECOMBINANT INSULIN

Human insulin was first manufactured at Genentech in 1978, by using recombinant DNA technology. Chemically, Insulin is a protein composed of two chains, an A chain (with 21 amino acids) and a B chain (with 30 amino acids), which are linked together by disulfide bonds.

12.6.1 Steps Involved in the Production of Insulin using Recombinant DNA Technology

- The human gene encoding insulin is isolated from the cell of islet of Langerhans.
- The plasmid DNA of the desired bacteria is cut out leading to the formation of an open plasmid ring. Escherichia coli (E. Coli) bacteria is widely used in producing human insulin but yeast may also be used.
- Thereafter, the gene encoding the recombinant protein is inserted into the open plasmid ring.

- Next step is transformation which involves inserting the resulting DNA back into the desired bacteria.
- The newly transformed bacterial cells are transferred into a suitable culture medium having all the essential nutrients required for the survival and growth of cells. As the bacterial cells reproduce by dividing, the human insulin gene is also reproduced in the newly created cells.
- This laboratory process is replicated at industrial scale leading to mass production of insulin. The science behind the mass production of biologically significant molecules is known as fermentation. For this, insulin producing recombinant cell are transferred into fermenters to produce them in large quantity.
- Finally, insulin secreted into the culture media is isolated and purified. This process involves the basic steps of separating the cells from culture or broth, increasing the concentration of the recombinant protein, purifying the recombinant product and finally assessing the quality of the product as explained in the introductory section.

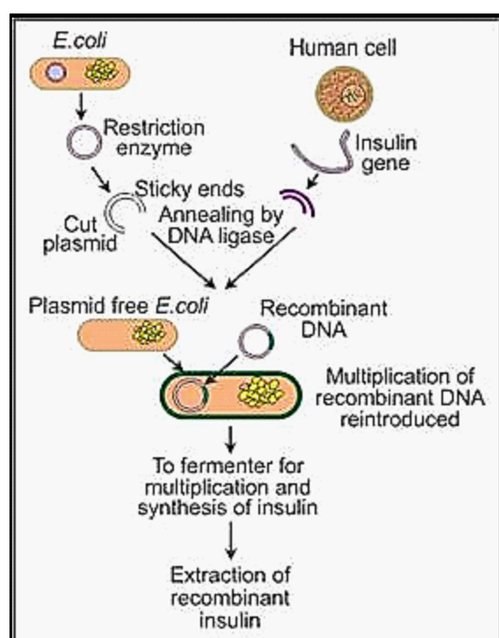


Fig. 12.5 *Steps Involved in the Production of Insulin by Recombinant DNA Technology*

12.6.2 Uses of Recombinant Insulin

The advantages of using recombinant insulin are as follows:

1. It can be used to treat patients suffering from severe diabetes.
2. It will eliminate the need to sacrifice animals for the production of recombinant insulin.

3. Recombinant insulin was not found allergic to patients when compare to insulin obtained from animals.
4. The cost of recombinant insulin is not very high.

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Check Your Progress

1. Define the recombinant vaccines.
2. What is recombinant DNA technology?
3. Elaborate on the subunit recombinant vaccines.
4. Illustrate the attenuated recombinant vaccines.
5. Define the vector recombinant vaccines.
6. Write two examples of tissue plasminogen activator produced by recombinant DNA technology.
7. Explain the role of a bioreactor.
8. Interpret the production of recombinant insulin.

12.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. In recent years, recombinant DNA technology has become a boon to produce new generation vaccines or novel recombinant vaccines. The recombinant vaccines may be broadly categorized into three categories namely: - subunit vaccines, attenuated recombinant vaccines, and vector recombinant vaccines.
2. Recombinant DNA technology refers to the joining together of genetic material, i.e., DNA molecules from two different species. The recombined DNA molecule is then inserted into a host organism to produce new genetic combinations that are of significant value to fields like medicine, science, agriculture, and other industry.
3. These vaccines are the essential components of the pathogenic organisms. Subunit vaccines include components of pathogenic organism like peptides, proteins, and DNA. The biggest advantage of using subunit recombinant vaccines is that they are pure, stable as well as safe to use.
4. In these types of vaccines, pathogenic organisms like viruses, bacteria, etc. are genetically modified to make them non-pathogenic for producing recombinant vaccines.
5. These vaccines are genetically modified viral vectors that can be used against certain pathogenic organism. For instance: - Vaccinia viruses is initially used by Jenner for the eradication of smallpox.

6. AITEPLASE and RETEPLASE are the examples of tissue plasminogen activator produced by recombinant DNA technology.
7. Genetically engineered host cells for production of recombinant proteins must be grown in large quantities. To achieve this, 'Bioreactors' are used for making pharmaceutical products such as antibiotics and insulin in large quantities.
8. Human insulin was first manufactured at Genentech in 1978, by using recombinant DNA technology. Chemically, Insulin is a protein composed of two chains, an A chain (with 21 amino acids) and a B chain (with 30 amino acids), which are linked together by disulfide bonds.

12.8 SUMMARY

- Recombinant DNA technology refers to the joining together of genetic material, i.e., DNA molecules from two different species. The recombined DNA molecule is then inserted into a host organism to produce new genetic combinations that are of significant value to fields like medicine, science, agriculture or industry.
- A bioreactor is a cylindrically shaped device used for carrying out a biological or biochemical reaction under controlled conditions of temperature, moisture, pH level, oxygen levels and stirring rate to yield the maximum cell growth and productivity.
- Batch bioreactor progressions involve filling the bioreactor with substantial amount of medium as well as the inoculums and working the bioreactor with no additional nutrients or medium until after the growth profile is complete.
- Fed-batch bioreactor progressions are the most common reactor processes used in the commercial setup. This methodology begins with a low starting volume and feeds nutrients as well as medium on a planned schedule without removing the harvest material.
- Stirred-Tank Reactors (STRs) are the most widely-used bioreactors and are well equipped with an impeller for homogenizing the culture media as well as a sparger for delivering the oxygen to the cells.
- Rocker bioreactors are offered as single-use systems. They comprises of a bag on a moving platform, relying on a rocking motion for the mixing.
- Recombinant DNA technology has become a boon to produce new generation vaccines or novel recombinant vaccines. The recombinant vaccines may be broadly categorized into three categories namely: - subunit vaccines, attenuated recombinant vaccines and vector recombinant vaccines.
- Subunit vaccines are very expensive as well as have the possibility of alteration in native conformation of the pathogen's subunit used for producing

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vaccines. For instance: - Hepatitis B mainly affects liver causing chronic hepatitis, cirrhosis and sometimes it can cause severe liver cancer too.

- The biggest advantage of using attenuated vaccines is that the original or native conformation of the antigenic determinants is preserved leading to highly effective immune response. For instance: - Intestinal disorder, Cholera, caused by Vibrio cholera is characterized by fever, abdominal pain or dehydration.
- The biggest advantage of using vaccinia virus for making recombinant vaccines are that they are harmless, stable, easy to use as well as can stimulate both branches of immunity, i.e., humoral and cell mediated immunity. Such recombinant vector vaccines have been developed successfully against fatal diseases such as herpes simplex virus, influenza, hepatitis, and rabies, etc.
- The most important application of recombinant DNA technology is the production of essential biomolecules like insulin, tissue plasminogen activators, blood products like clotting factor VIII, albumins, cytokines, follicle stimulating hormones, and interferon, etc.
- Tissue plasminogen activator is the first pharmaceutical product produced using mammalian cell culture at Genentech in 1982. Tissue plasminogen activator is a protease enzyme which helps in dissolving blood clots.
- Human insulin was first manufactured at Genentech in 1978, by using recombinant DNA technology. Chemically, Insulin is a protein composed of two chains, an A chain (with 21 amino acids) and a B chain (with 30 amino acids), which are linked together by disulfide bonds.

12.9 KEY WORDS

- **Recombinant DNA technology:** Recombinant DNA technology refers to the joining together of genetic material, i.e., DNA molecules from two different species.
- **Subunit recombinant vaccines:** These vaccines are the essential components of the pathogenic organisms. Subunit vaccines include components of pathogenic organism like peptides, proteins, and DNA.
- **Attenuated recombinant vaccines:** In these types of vaccines, pathogenic organisms like viruses, bacteria, etc., are genetically modified to make them non-pathogenic for producing recombinant vaccines.
- **Vector recombinant vaccines:** These vaccines are genetically modified viral vectors that can be used against certain pathogenic organism. For instance: - Vaccinia viruses is initially used by Jenner for the eradication of smallpox.

- **Application of recombinant DNA technology:** The most important application of recombinant DNA technology is the production of essential biomolecules like insulin, tissue plasminogen activators, blood products like clotting factor VIII, albumins, cytokines, follicle stimulating hormones, and interferon, etc.
- **Tissue plasminogen activator:** Tissue plasminogen activator is the first pharmaceutical product produced using mammalian cell culture at Genentech in 1982. Tissue plasminogen activator is a protease enzyme which helps in dissolving blood clots.
- **Recombinant insulin:** Human insulin was first manufactured at Genentech in 1978, by using recombinant DNA technology. Chemically, Insulin is a protein composed of two chains, an A chain (with 21 amino acids) and a B chain (with 30 amino acids), which are linked together by disulfide bonds.

12.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the recombinant vaccines.
2. What is recombinant DNA technology?
3. Define the subunit recombinant vaccines.
4. Interpret the attenuated recombinant vaccines.
5. State the vector recombinant vaccines.
6. Write two examples of tissue plasminogen activator produced by recombinant DNA technology.
7. Elaborate on the role of a bioreactor.
8. Illustrate the production of recombinant insulin.

Long-Answer Questions

1. Define recombinant vaccines. Describe deferent types of vaccines.
2. Explain the production of pharmaceutically essential compounds. Give appropriate examples.
3. With the help of a diagram explain the production of hepatitis B virus surface antigen particle in yeast cells.
4. Briefly discuss the production of recombinant tissue plasminogen activator.
5. Write down the steps involved in the production of recombinant tissue plasminogen activator.

6. What are the uses of recombinant tissue plasminogen activator?
7. Explain the process of producing recombinant insulin.
8. Analyse the uses of recombinant insulin.

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12.11 FURTHER READINGS

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UNIT 13 GENE THERAPY

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Structure

13.0 INTRODUCTION

Gene therapy is a medical field which focuses on the genetic modification of cells to produce a therapeutic effect or the treatment of disease by repairing or reconstructing defective genetic material. The first attempt at modifying human DNA was performed in 1980 by Martin Cline, but the first successful nuclear gene transfer in humans, approved by the National Institutes of Health, was performed in May 1989. The first therapeutic use of gene transfer as well as the first direct insertion of human DNA into the nuclear genome was performed by French Anderson in a trial starting in September 1990. It is thought to be able to cure many genetic disorders or treat them over time.

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Molecular imaging is a rapidly emerging technique originated from the field of radiology from a need to better understand fundamental molecular processes inside organisms in a non-invasive manner. It is a non-invasive, real time approach which involves visualization, measurement of physiological or pathological process in the living organism at the cellular as well as molecular level. This is in contrast to conventional methods for obtaining molecular information from preserved tissue samples, such as histology. Also, this technique involves repetitive studies in the same being, as a result making it feasible to gather longitudinal data and decrease the number of animals in addition to cost. Hence, molecular imaging plays a significant role in accurate diagnosis, easier detection as well as research and development in the field of drug and discovery.

Molecular imaging techniques requires high resolution and high sensitive instruments to detect specific imaging agents or molecules of interest that may be either ones produced naturally by the body, or synthetic molecules produced in a laboratory and injected into a patient by a doctor. The function of these high end instruments involves linking the imaging signal with molecular event. Improvement in instruments and iterative image reconstruction has resulted in high resolution images that reveal even minute injury and realize precise quantification of biological progression. There are five different kind of imaging techniques available carrying out the process of molecular imaging namely, X-ray, Computed Tomography (CT) imaging, radionuclide imaging (involving PET and SPECT), Optical Imaging (OI), Magnetic Resonance Imaging (MRI), and UltraSound (US) imaging.

In this unit, you will study about the gene therapy, somatic gene therapy, germline gene therapy, applications of advanced techniques, positron emission tomography, computed tomography, molecular imaging, and human genome project.

13.1 OBJECTIVES

After going through this unit, you will be able to:

- Comprehend the concept of gene therapy
- Understand the somatic gene therapy
- Explain the germline gene therapy
- Define the positron emission tomography
- Elaborate on the computed tomography
- Interpret the molecular imaging
- Analyse the human genome project

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13.2 GENE THERAPY

The methodology of gene therapy initially developed in 1972 refers to the procedure where a foreign DNA containing a functional gene is introduced into a patient to treat a genetic disorder. In the process, DNA is carefully chosen to rectify the effect of a mutated gene that is causing genetic disease like cystic fibrosis and muscular dystrophy. On the basis of cell type treated, gene therapy is of two types:

1. Somatic Gene Therapy

It refers to the transfer of a section of functional DNA to any somatic cell of the body that doesn't produce any gamete, i.e., either sperm or eggs. Thus, the effects of somatic gene therapy will not be passed onto the patient's offspring.

2. Germline Gene Therapy

It refers to the transfer of a section of functional DNA to gametes, i.e., either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.

13.2.1 Gene Therapy Techniques

There are several ways for carrying out the gene therapy. These include:-

A. Gene Augmentation Therapy

- Gene augmentation therapy is generally used to treat disorders caused by a mutation that prevents a gene from producing a functional protein product.
- This therapy aims at adding the functional version of the DNA back into the cell.
- The added new gene produces a functional product at optimum levels to restore the function of the protein that was missing earlier.
- However, the technique is successful only up to the limited extent, i.e., only if the effects of the disease are reversible
- This technique has been used to treat loss of function disorders such as cystic fibrosis.

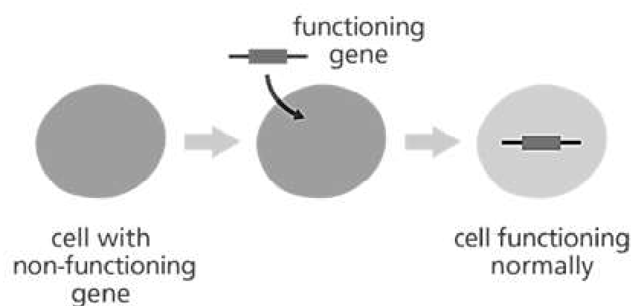


Fig. 13.1 *Principle behind Gene Augmentation Therapy*

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B. Gene Inhibition Therapy

- Gene inhibition therapy is suitable and effective for the treatment of infectious diseases such as cancer and inherited disease caused by inappropriate gene activity.
- Gene inhibition therapy aims at introducing a gene which either inhibits the expression of another gene or interferes with the activity of end product of some other gene.
- The fundamental basis of gene inhibition therapy is to remove or reduce the activity of a gene that promotes the growth of disease-related cells.
- For example, over activity of growth promoting cells is responsible for deadly disease like cancer. Hence, gene inhibition therapy aims at eliminating the activity of those over growing cells.

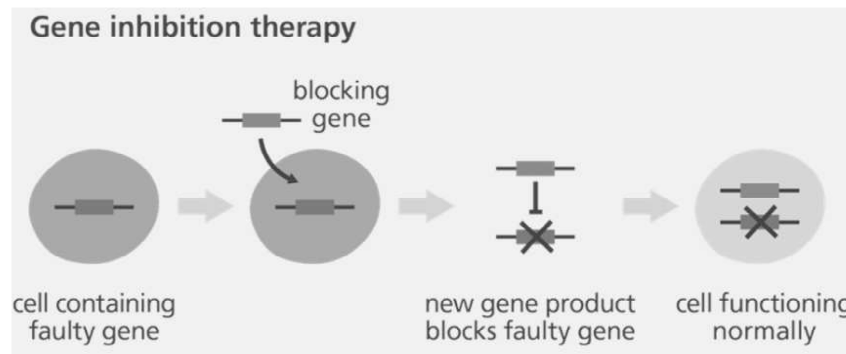


Fig. 13.2 *Principle behind Gene Inhibition Therapy*

C. Killing of Specific Cells

- Killing of specific cells methodology aims to insert foreign DNA into a diseased cell in such a way that it causes that particular cell to die.
- This can be attained in one of two ways:
  - (i) The inserted foreign DNA must contains a “Suicide” gene that makes a highly toxic product which can kill the diseased cell
  - (ii) The inserted foreign DNA must express a protein that marks the diseased cells in such a way that it is attacked automatically by the body’s natural immune system.
- However, the foreign DNA should be inserted in a specific manner to prevent the death of normally functioning cells.
- This methodology is most suitable for diseases like cancer that can be treated effectively by destroying certain groups of cells.

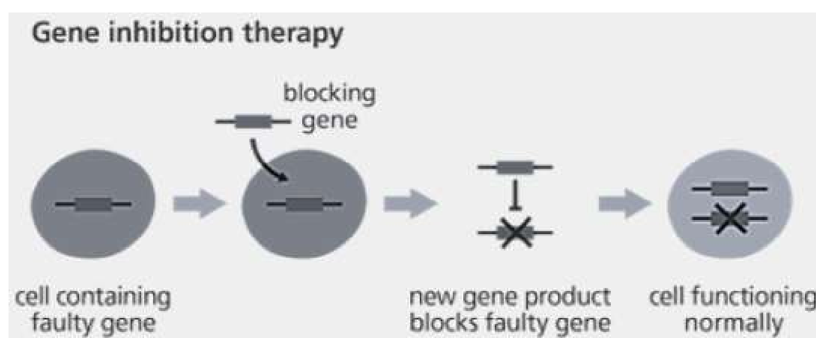


Fig. 13.3 *Principle behind Killing Specific Cell Gene Therapy*

13.2.2 Challenges of Gene Therapy

- To ensure that new foreign DNA reaches to the target cell and be effective as delivering to the wrong cell would be harmful and can cause serious disorders.
- Even on reaching the target cell, right cell needs to be turned on as the body's natural response is to obstruct this process by shutting down genes that are showing some unusual activity.
- Foreign DNA is a potential intruder and can spark an immune response which could lead to autoimmune disorders. Thus, it is challenging to introduce foreign DNA into the patient's body without the immune system 'Noticing'.
- Further, scientist should make sure that the foreign DNA does not interrupt the function of other normally functioning genes. For instance, if the foreign DNA interferes with an important gene involved in the regulation of cell division, then it could potentially result in serious disorder like cancer.
- Gene therapy is a new age approach and often requires an individual, case-by-case approach. This approach may be effective in long run, but is very expensive.

13.3 MOLECULAR IMAGING

Molecular imaging is a rapidly emerging technique originated from the field of radiology from a need to better understand fundamental molecular processes inside organisms in a non-invasive manner. It is a non-invasive, real time approach which involves visualization, measurement of physiological or pathological process in the living organism at the cellular as well as molecular level. This is in contrast to conventional methods for obtaining molecular information from preserved tissue samples, such as histology. Also, this

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technique involves repetitive studies in the same being, as a result making it feasible to gather longitudinal data and decrease the number of animals in addition to cost. Hence, molecular imaging plays a significant role in accurate diagnosis, easier detection as well as research and development in the field of drug and discovery.

Molecular imaging techniques requires high resolution and high sensitive instruments to detect specific imaging agents or molecules of interest that may be either ones produced naturally by the body, or synthetic molecules produced in a laboratory and injected into a patient by a doctor. The function of these high end instruments involves linking the imaging signal with molecular event. Improvement in instruments and iterative image reconstruction has resulted in high resolution images that reveal even minute injury and realize precise quantification of biological progression.

There are five different kind of imaging techniques available carrying out the process of molecular imaging namely, X-ray, Computed Tomography (CT) imaging, radionuclide imaging (involving PET and SPECT), Optical Imaging (OI), Magnetic Resonance Imaging (MRI), and UltraSound (US) imaging.

The definitive aim and objective of molecular imaging techniques is to enable monitoring of the physiological and biochemical processes happening inside a living organism in real time. Modern research in molecular imaging involves cellular/ molecular biology, chemistry, and medical physics, and is focused on several aspects such as:

- Developing imaging procedures to identify molecules which were previously undetectable.
- Expanding the number as well as types of contrast agents available for carrying out the molecular imaging.
- Developing several functional contrast agents that give details regarding the diverse activities that cells as well as tissues execute in both health and disease.

13.4 CT-SCAN

CT-SCAN refers to the Computerized Tomography (CT) scan which combines a series of X-ray images obtained from different angles around your body and then utilizes computer processing to generate cross-sectional images (slices) of the blood vessels, soft tissues and bones inside your body. As it combines images from several angles, CT scan images provide much-detailed information when compared to plain X-rays. A CT scan can be utilized to visualize almost all body parts and is used to detect injury or disease as well as to plan surgical, medical or radiation treatment.

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Fig. 13.4 *Radiologist Talking to an Examinee Undergoing CT-SCAN*

13.4.1 Why to do CT-SCAN

CT scan helps to:

- Detect bone and muscle disorders like bone tumours and fractures.
- Identify the position of a tumour, infection or blood clot.
- Guiding procedures like biopsy, surgery, and radiation therapy.
- Monitor diseases as well as conditions like heart disease, liver masses cancer, and lung nodules.
- Monitor the effectiveness of certain treatments like cancer treatment.
- Detect internal bleeding and internal injuries.

13.4.2 Risks Associated with CT-SCAN

- Radiation Exposure: A person is briefly exposed to ionizing radiation during CT-SCAN. The amount as well as duration of exposure to radiations is longer than the normal X-ray. However, research shows that CT-Scan does not cause long-term harm to the patient. Also, newer, faster machines and techniques require less radiation than was previously used.
- Damage to Unborn Babies: Even though the radiation from a CT scan is not likely to harm unborn baby, doctor may suggest other type of potentially safe test like ultrasound in order to avoid exposing unborn baby to radiation.
- Reactions to Contrast Material: Sometimes the contrast agent given via oral or rectal or intravenous mode can cause allergic reactions or medical

complications. However, allergic reactions are mild and consequently results in a red rash or itchiness. In rare instances, an allergic reaction can be serious that can cause life-threatening conditions.

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13.4.3 Preparation before Going for a CT-SCAN

Depending on the body part, a patient is asked to:

- Wear a hospital gown while the procedure.
- Remove all metal objects, such as earrings, jewellery, eyeglasses, belt, and dentures.
- Refrain from eating or drinking for a few hours before going for the CT-Scan.

13.4.4 Contrast Material used for Carrying CT-SCAN

A special dye referred to as contrast material is required for some CT scans to highlight the areas of the body under examination. The contrast material blocks X-rays and appears white on molecular images or scan, which can assist in emphasizing the blood vessels, intestines or other structures. Contrast material might be given via different mode:

(i) By mouth- This method involves swallowing a liquid that contains contrast material for scanning and examining body parts like esophagus or stomach.

(ii) By injection- **This method involves injecting the** contrast agents intravenously to help can and examine liver or blood vessels, urinary tract and gallbladder.

(iii) By enema- This method involves inserting the contrast agent in rectum to enable clear scanning and examining of intestine.

13.4.5 CT-SCAN Procedure

CT scanners are shaped like a large doughnut standing on its side. Person undergoing scan lie down on a narrow, motorized (machine driven) table that slides through the opening into a tunnel. Straps and pillows are often used to help the person stay in position comfortably. During a head scan, the table is generally fitted with a special cradle that keeps the head still in position.

As the table slides the Peron into the CT scanner, detectors and X-ray tube rotate around the person undergoing examination. Each rotation yields several images of thin slices of the body part being examined.

A technologist sitting in a separate room monitors the person. The person undergoing examination can easily communicate to the technologist. The radio technologist might ask the examinee to hold breath at certain points in order to avoid blurring the images.

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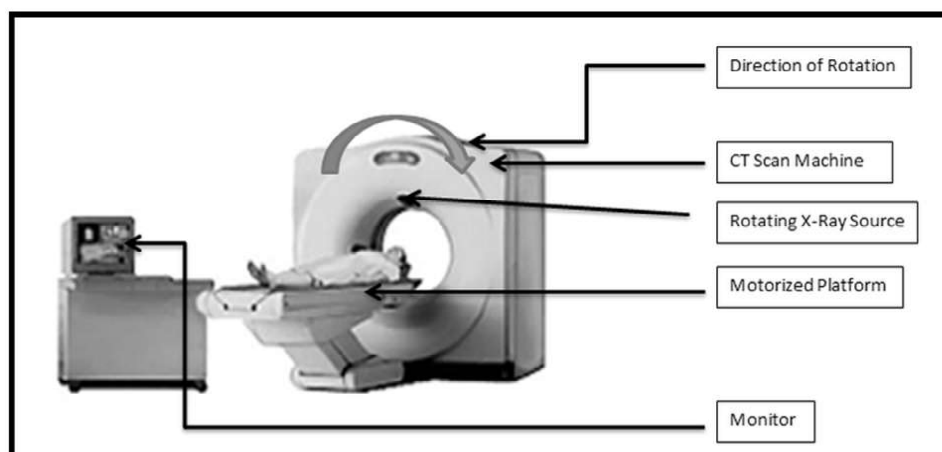


Fig. 13.5 *Parts of the CT-SCAN Machine*

Duration of CT-SCAN

CT scans are painless and, with new sophisticated instruments and takes about 30 minutes.

Post - CT-SCAN

Person undergoing CT-SCAN can immediately return to normal routine after the procedure. Doctors recommend drinking a lot of fluids to remove the contrast material from patient's body (in case it is given).

Results

CT-Scan images are stored up as electronic data files and are reviewed on a computer screen by a radiologist. The final interpretation is reviewed and signed by a doctor.

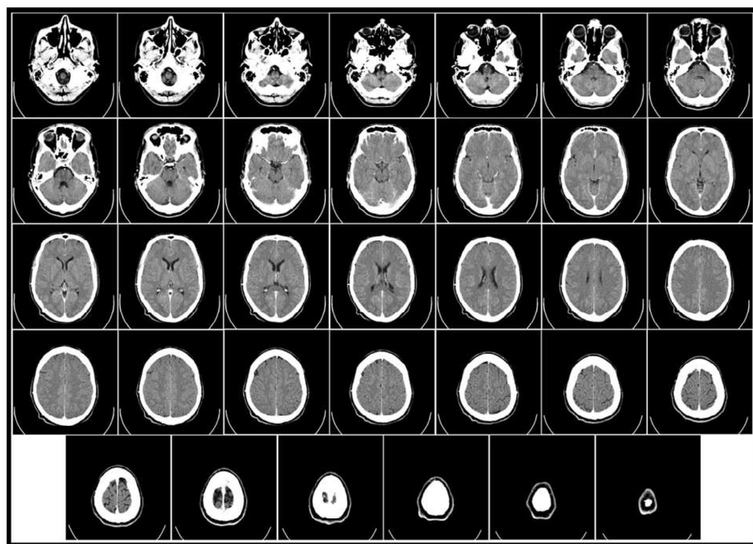


Fig. 13.6 *CT-Scan of Human Head Capture using Intravenous Contrast Agent*

13.5 PET- SCAN

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PET referring to the Positron Emission Tomography (PET) scan is an imaging test that divulges how your tissues and organs are functioning inside by using a radioactive drug (tracer) to show this activity. This scan can help to detect disease ahead of other imaging tests. The radioactive tracer may be swallowed orally or injected intravenously, or inhaled, depending on the type of organ or tissue under consideration. While examining, the radioactive tracer keeps on collecting in areas of the body that exhibit higher levels of chemical activity that generally correspond to areas of disease. On a PET scan, these diseased areas show up as bright spots.

The advantage of PET scan is that it helps in revealing diseased conditions like heart disease, cancers and brain disorders. Often, PET images are combined along with other molecular imaging techniques such as CT or MRI, i.e., PET-CT or PET-MRI scans to form special views.

13.5.1 Why to do PET-SCAN

A PET-SCAN helps in identifying body conditions like heart disease, cancers and brain disorders. Often, PET images are combined along with other molecular imaging techniques such as CT or MRI, i.e., PET-CT or PET-MRI scans to form special views to assess condition.

A. Cancer

On a PET-SCAN, cancer cells show up as bright spots as these cells have a higher metabolic rate when compared to normal cells. PET scans may be useful in:

- Help in detecting cancer
- Revealing whether cancer is metastatic, i.e., spreading or not
- Assessing whether a particular cancer treatment is working or not
- Finding a cancer recurrence

However, sometimes non-cancerous conditions start looking like cancer, hence these PET-SCAN images needs to be thoroughly interpreted by the specialist. Moreover, some cancers do not appear on PET scans like:

- Cervical Cancer
- Brain Cancer
- Oesophageal Cancer
- Head and neck cancer

- Colorectal cancer
- Lung cancer
- Thyroid cancer
- Melanoma cancer
- Lymphoma cancer
- Prostate cancer
- Pancreatic cancer

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B. Heart Disease

PET scans images can also reveal areas of decreased blood flow in the heart. This might enable the doctor to decide whether the patient benefits from angioplasty or bypass surgery.

C. Brain Disorders

PET scan images can also help to evaluate certain brain disorders like Alzheimer's disease, tumours and seizures.

Risks Associated with PET-SCAN

A radioactive drug (tracer) is used to obtain the PET-SCAN images, even though the patient is exposed to very low amount of radiation, yet the tracer might:

- Cause a severe allergic reaction.
- Expose unborn baby to radiation if case the patient is pregnant.
- Expose the child to radiation in case the patient is breast-feeding.
- In order to avoid some risks, please inform doctor:-
- About the allergies patient is suffering
- Whether the patient has any other medical condition
- Whether the patient is on any specific diet
- Whether the patient is pregnant
- Whether the patient is breast-feeding
- Whether the patient is claustrophobic, i.e., afraid of enclosed spaces

Doctors generally ask the patient to avoid heavy exercise a couple of days before the scan as well as to stop eating a few hours before the test.

13.5.2 PET-SCAN Procedure

The PET scanner is a huge machine that appears like a giant doughnut standing upright. In some centres, a combined CT-PET scanner is used to carry out the

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scan. The entire procedure takes about two hours. The doctor asks the patient to empty the bladder before the test. Then, the patient is given a radioactive drug (tracer) either orally, intravenously or via inhalation. The patient needs to wait 30 to 60 minutes before the tracer is absorbed by the body.

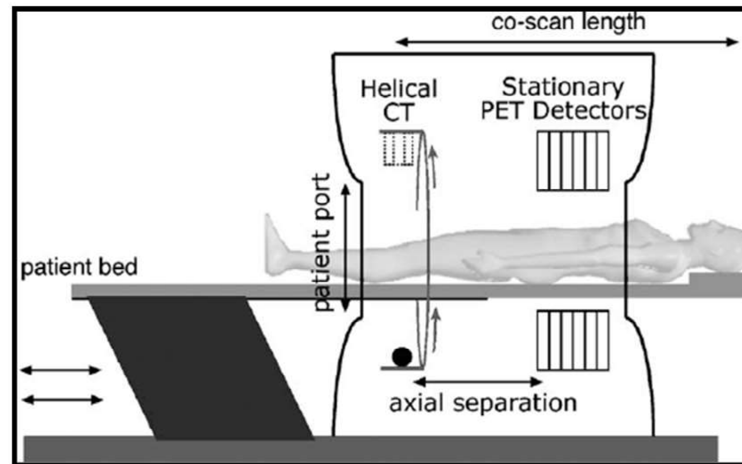


Fig. 13.7 *PET- Scanner*

13.5.3 During the PET- SCAN Procedure

The Patient is asked to lie on a narrow, padded table that slides into the scanner. During the entire scan of about 30 minutes, patient needs to be in still position to prevent blur images. In case, patient is having a CT and PET scan in the same machine during the same appointment then CT scan will be done first.

13.5.4 Post - PET-SCAN

Person undergoing PET-SCAN can immediately return to normal routine after the procedure. Doctors recommend drinking a lot of fluids to remove the radioactive tracer from patient's body.

13.5.5 Results

The radioactive tracer keeps on collecting in areas of the body that exhibit higher levels of chemical activity that generally correspond to areas of disease. On a PET scan image, these diseased areas show up as bright spots. A specialist interprets the scan images carefully. The radiologist might compare the PET images with other tests like Computerized Tomography (CT) or Magnetic Resonance Imaging (MRI) to conclude.

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Fig. 13.8 *CT-Scan, PET-Scan and a Combined CT-PET Scan*

13.6 ADVANTAGES OF MOLECULAR IMAGING

- It exhibits the degree of the situation and will identify if the disease has spread to other parts of the patient's body or not.
- It enables the doctor to decide the most suitable and effective therapy based on the unique biologic characteristics of the patient.
- It enables the specialist to evaluate the patient's body reactions to specific drugs that may be used for treatment.
- It enables the specialist to accurately establish whether or not a treatment regimen would be successful.
- It can help to quickly adapt treatment plans whenever there are modifications in the patient's cellular activity.
- It also helps to makes an accurate estimation of disease progression.
- Molecular imaging also helps in identifying whether or not a disease has recurred and thus can be helpful in managing future care of the patient.

All these advantages of molecular imaging gives the technology an upper hand and thus is quite popular with specialist as such detailed information is not possible with other traditional diagnostic methods. Thus, to conclude molecular imaging is painless, non-invasive and safe. It might be used to both diagnose and treat diseases such as Alzheimer's disease bone, disorders cancer, thyroid and kidney disorders, Parkinson's disease, and lung disorders, etc.

13.7 HUMAN GENOME PROJECT

The Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy (DOE) and the National Institutes of Health. This project was started in 1990 and completed in 2003. During the early years of the Human Genome Project, the Welcome Trust (U.K.) happened to be the major

partner; further contributions came from Japan, France, Germany, China, and others.

13.7.1 History of Human Genome Project

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The timeline below shows the history of Human Genome Project:

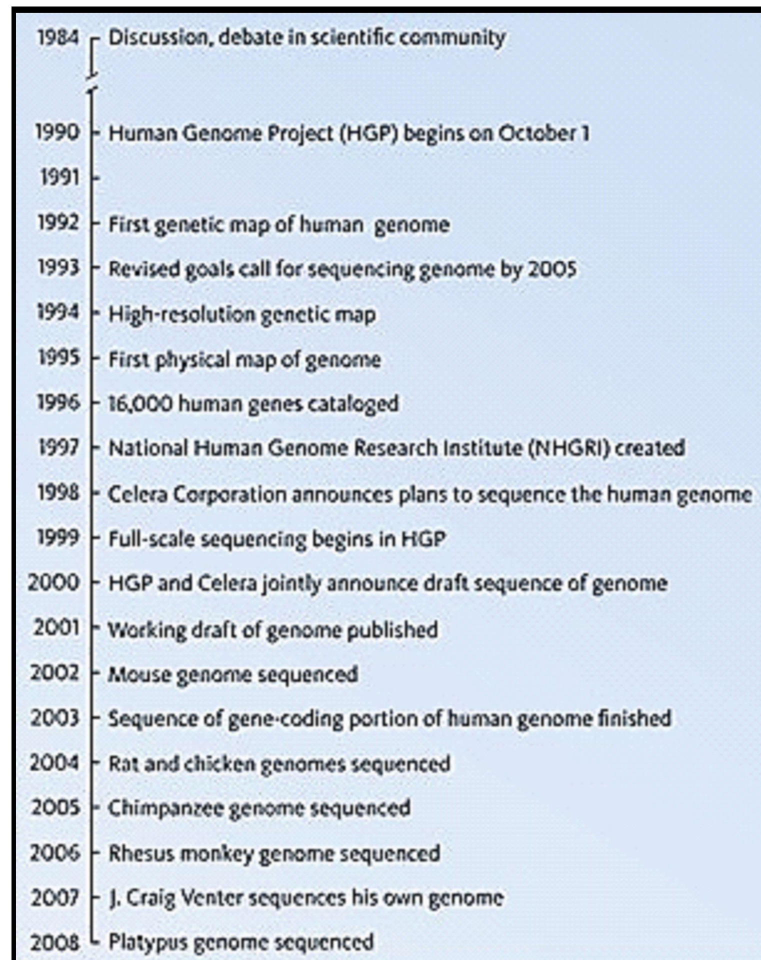


Fig. 13.9 *Timeline of the Human Genome Project*

13.7.2 Aims and Objectives of the Human Genome Project

The following were the aims and objectives of the project:

- To identify all the approximately 20,500 genes present in human DNA.
- To determine the DNA sequences of three billion chemical base pairs that make up human DNA.
- To store the obtained information in databases for future use.
- To improve tools for data analysis and interpretation.
- To transfer related technologies to the private sector for future research and development purpose.

- To address all the Ethical, Legal, as well as Social Issues (ELSI) that may arise from the human genome project.

13.7.3 Mapping of the Human Genome

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In this project, two different methodologies are used:

Before beginning a Human Genome Sequencing project, it was a pre-requisite to generate a good framework map. Two common processes were developed for mapping human genome was:-

A. Standard Method

The standard method involves looking for a segment of the genome and positioning where it belongs. Genetic maps which are based on recombination frequencies between markers are helpful in ordering genes. Molecular markers such as VNTRs (Microsatellites), STSs, RFLP and SNPs have been used in mapping human genome.

B. Whole Genome Short-Gun Method

The whole genome shotgun sequencing methodology involves shearing of genomic DNA followed by cloning, to produce a genomic library. The above step is followed by random sequencing of cloned DNA fragments followed by shotgun assembly, i.e., the assembly of the fragment sequences into larger units on the basis of their overlaps. Groups of cloned DNA segments that can be aligned in an overlapping fashion to cover a region of the human genome are referred to as contains. Initially, YACs, i.e., Yeast Artificial Chromosomes were used followed by BACs, i.e., Bacterial Artificial Chromosomes.

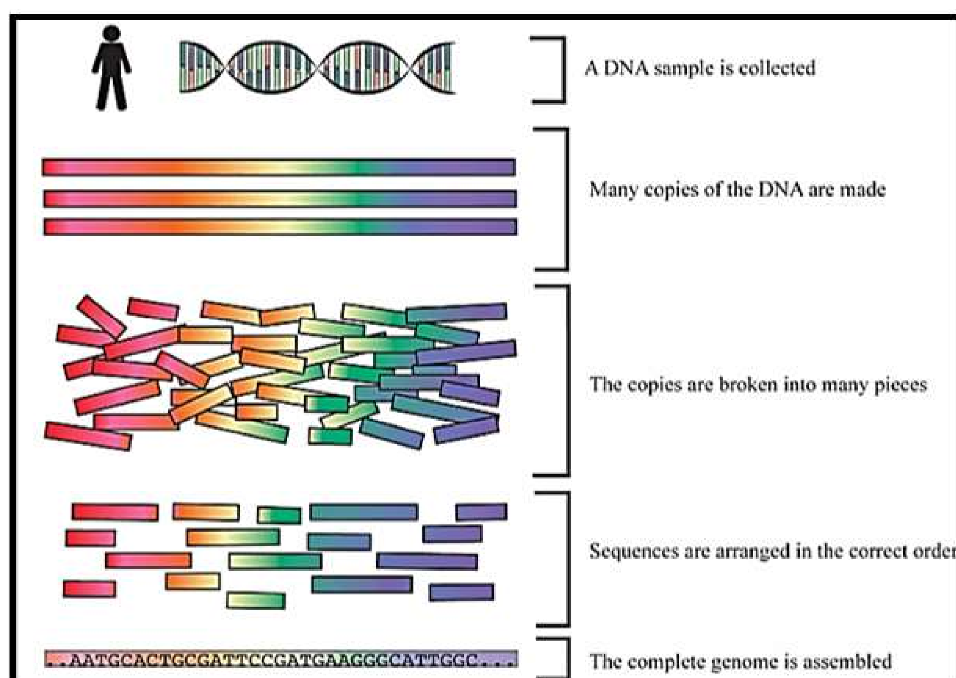


***Fig. 13.10** Shotgun Whole Genome Sequencing*

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13.7.4 The Process of the Human Genome Project

The whole process can be summarized as follows:

- To carry out the Human Genome Project, Researchers collected blood (female) or sperm (male) samples from different races like African, American (North, Central, South), European, and Asian ancestry and then a few samples were processed as DNA resources.
- The complete gene set was isolated from a cell.
- Chromosomes, which range in size from 50 million to 250 million bases, split into smaller fragments.
- This DNA structure was then amplified with the help of a vector which mostly was BAC (Bacterial artificial chromosomes) and YAC (Yeast artificial chromosomes).
- The smaller fragments were then sequenced using DNA sequencers.
- On the basis of overlapping regions, the sequences were then arranged.
- All the information of this genome sequence was then stored in a computer-based program.
- This way the entire genome was sequenced and stored as genome database in computers.
- Finished sequence is submitted to major public sequence databases.
- Submission of the Human Genome Project data ensured the free availability of sequence data to everyone around the world.

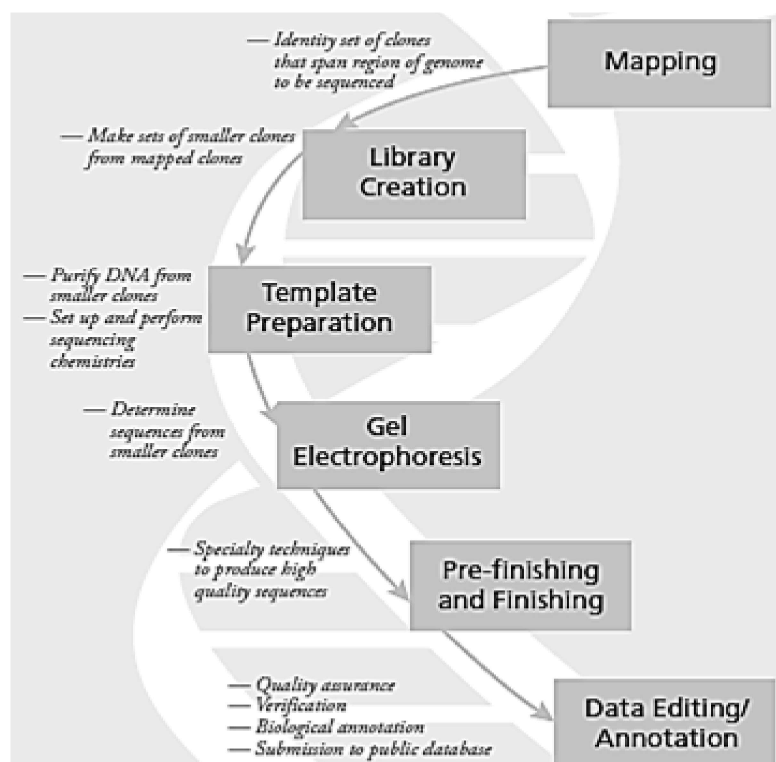


Fig. 13.11 *DNA Sequencing Process*

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13.7.5 Features of the Human Genome Project

Salient features of the Human Genome Project are as follows:

- The human genome consists of 3164.7 million chemical nucleotide bases (A, C, T and G).
- Almost all (99.9%) nucleotide bases are exactly the same in all people.
- On an average, a gene is made up of 3000 nucleotides.
- However, the size of the gene vary greatly, largest known human gene is "Dystrophin" – having around 2.4 million bases.
- Approximately, 30,000 genes are estimated.
- The function of more than 50 percent of the genes is yet to be discovered.
- Proteins are coded by less than 2 percent of the genome.
- Most of the genome is made up of repetitive sequences referred to as junk DNA which have no specific coding purpose.
- However, such redundant codes can help in better understanding of genetic development of humanity through the ages.
- A-T rich regions are gene-poor.
- G-C rich regions are gene-dense.
- Chromosome-I has the most genes (2968).
- Y chromosome present in males has the fewest number of genes (231).
- Scientists have identified about 1.4 million locations where single base DNA differences (SNPs) occur in human.
- SNPs will help to localize the disease associated sequences in the chromosomes.
- Finding the DNA sequences underlying such common diseases as cardiovascular disease, diabetes, arthritis and cancers is being aided by human variation maps (SNPs) generated in HGP.

13.7.6 Applications of HGP

Scientists discovered that chromosomes in the human population differ at about 0.1%. Understanding these minor differences could lead to discovery of heritable diseases. Information obtained from the HGP has already lead to positive discoveries in health care as well as research and development. The most publicized successes comprise the cloning of genes accountable for diseases such as retinoblastoma, Duchenne muscular dystrophy, cystic fibrosis and neurofibromatosis. It has also aided researchers in looking for genes connected with, types of inherited colon cancer, fragile X- syndrome, familial breast cancer and Alzheimer's disease.

Current and potential applications of genome research will address national needs:

1. Molecular Medicine

Genetic research will help the scientist to look into the fundamental reasons behind disorders rather than just concentrating on treating the symptoms. Genetic screening will increase the rapidity and specificity of the diagnostic tests enabling early detection and control of disorders. Medical researchers will also be able to produce therapeutic products based on new classes of immunotherapy techniques, drugs as well as potential expansion or substitution of faulty genes through gene therapy.

2. Waste Control and Environmental Cleanup

In 1994, through advances gained by the Human Genome Project, the DOE devised the 'Microbial Genome Initiative' to sequence the genomes of bacteria helpful in the fields such as environmental remediation, energy production, industrial processing, and toxic waste reduction, etc. Six microbes that live under unfavourable environmental conditions such as extreme temperature and pressure conditions have been sequenced. By learning the unique protein structure of these six microbes flourishing under extreme conditions, may enable the researchers to use the organisms and their enzymes for realistic function like environmental cleanup and waste control.

3. Biotechnology

Human Genome Project has significantly stimulated investment by large corporations to promote the research and development in the field of biotechnology.

4. Energy Sources

Biotechnology, strengthened by the HGP, will pave the way in improvising the utilization of fossil-based resources. Biotechnology will help addressing the ever increasing energy demands by providing a cleaner means for the bioconversion of raw materials to refined products.

13.7.7 Ethical, Legal and Social Implications Human Genome Project

The DOE and NIH genome programs each set aside approximately 3-5% of their annual budgets for the study of ELSI. Four major priorities being addressed by ELSI:

I. Issue of privacy and Confidentiality in the use as well as interpretation of data obtained by the HGP.

II. The second main concern for ELSI is the clinical integration of new genetic technologies.

III. The third issues include the commercialization of the products obtained from data. This issue aims at resolving queries related to ownership of tissue and tissue derived products, copyrights, and patents, etc.

IV. Last but not the least is related to the education and awareness of the health professionals regarding new technologies.

NOTES

Check Your Progress

1. What do you understand by the gene therapy?
2. Explain the somatic gene therapy.
3. Define the germline gene therapy.
4. Illustrate the gene inhibition therapy.
5. Elaborate on the molecular imaging.
6. What is CT scan?
7. Define the PET scan.
8. Interpret the human genome project.
9. Explain the applications of HGP.

13.8 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. The methodology of gene therapy initially developed in 1972 refers to the procedure where a foreign DNA containing a functional gene is introduced into a patient to treat a genetic disorder.
2. It refers to the transfer of a section of functional DNA to any somatic cell of the body that doesn't produce any gamete, i.e., either sperm or eggs. Thus, the effects of somatic gene therapy will not be passed onto the patient's offspring.
3. It refers to the transfer of a section of functional DNA to gametes, i.e., either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.
4. Gene inhibition therapy is suitable and effective for the treatment of infectious diseases such as cancer and inherited disease caused by inappropriate gene activity.
5. Molecular imaging is a rapidly emerging technique originated from the field of radiology from a need to better understand fundamental molecular processes inside organisms in a non-invasive manner. This is in contrast to conventional methods for obtaining molecular information from preserved tissue samples, such as histology. Molecular imaging plays a significant role in accurate diagnosis, easier detection as well as research and development in the field of drug and discovery.
6. CT-SCAN refers to the Computerized Tomography (CT) scan which combines a series of X-ray images obtained from different angles around your body and then utilizes computer processing to generate cross-sectional images (slices) of the blood vessels, soft tissues and bones inside your body.

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7. PET referring to the Positron Emission Tomography (PET) scan is an imaging test that divulges how your tissues and organs are functioning inside by using a radioactive drug (tracer) to show this activity.
8. The Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy (DOE) and the National Institutes of Health. This project was started in 1990 and completed in 2003. During the early years of the Human Genome Project, the Welcome Trust (U.K.) happened to be the major partner; further contributions came from Japan, France, Germany, China, and others.
9. Scientists discovered that chromosomes in the human population differ at about 0.1%. Understanding these minor differences could lead to discovery of heritable diseases. Information obtained from the HGP has already lead to positive discoveries in health care as well as research and development.

13.9 SUMMARY

- The methodology of gene therapy initially developed in 1972 refers to the procedure where a foreign DNA containing a functional gene is introduced into a patient to treat a genetic disorder.
- Somatic gene therapy refers to the transfer of a section of functional DNA to any somatic cell of the body that doesn't produce any gamete, i.e., either sperm or eggs.
- Germline gene therapy refers to the transfer of a section of functional DNA to gametes, i.e., either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.
- Gene augmentation therapy is generally used to treat disorders caused by a mutation that prevents a gene from producing a functional protein product.
- Gene inhibition therapy is suitable and effective for the treatment of infectious diseases such as cancer and inherited disease caused by inappropriate gene activity.
- Killing of specific cells methodology aims to insert foreign DNA into a diseased cell in such a way that it causes that particular cell to die.
- Molecular imaging is a rapidly emerging technique originated from the field of radiology from a need to better understand fundamental molecular processes inside organisms in a non-invasive manner.
- CT-SCAN refers to the Computerized Tomography (CT) scan which combines a series of X-ray images obtained from different angles around your body and then utilizes computer processing to generate cross-sectional images (slices) of the blood vessels, soft tissues and bones inside your body.
- PET referring to the Positron Emission Tomography (PET) scan is an imaging test that divulges how your tissues and organs are functioning inside by

using a radioactive drug (tracer) to show this activity. This scan can help to detect disease ahead of other imaging tests.

- The Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy (DOE) and the National Institutes of Health. This project was started in 1990 and completed in 2003.

13.10 KEY WORDS

- **Gene therapy:** The methodology of gene therapy initially developed in 1972 refers to the procedure where a foreign DNA containing a functional gene is introduced into a patient to treat a genetic disorder.
- **Somatic gene therapy:** It refers to the transfer of a section of functional DNA to any somatic cell of the body that doesn't produce any gamete, i.e., either sperm or eggs.
- **Germline gene therapy:** It refers to the transfer of a section of functional DNA to gametes, i.e., either eggs or sperm. Thus, the effects of gene therapy will be passed onto the patient's offspring as well as subsequent generations.
- **Gene inhibition therapy:** Gene inhibition therapy is suitable and effective for the treatment of infectious diseases such as cancer and inherited disease caused by inappropriate gene activity.
- **Molecular imaging:** Molecular imaging is a rapidly emerging technique originated from the field of radiology from a need to better understand fundamental molecular processes inside organisms in a non-invasive manner.
- **CT-scan:** CT-SCAN refers to the Computerized Tomography (CT) scan which combines a series of X-ray images obtained from different angles around your body and then utilizes computer processing to generate cross-sectional images (slices) of the blood vessels, soft tissues and bones inside your body.
- **PET- scan:** PET referring to the Positron Emission Tomography (PET) scan is an imaging test that divulges how your tissues and organs are functioning inside by using a radioactive drug (tracer) to show this activity.
- **Human genome project:** The Human Genome Project (HGP) was a 13-year project coordinated by the U.S. Department of Energy (DOE) and the National Institutes of Health. This project was started in 1990 and completed in 2003.

13.11 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Elaborate on the gene therapy.
2. Define the somatic gene therapy.

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3. Explain the germline gene therapy.
4. Interpret the gene inhibition therapy.
5. State the molecular imaging.
6. What do you understand by the CT scan?
7. Define the PET scan.
8. Illustrate the human genome project.
9. Explain the applications of HGP.

Long-Answer Questions

1. Discuss briefly the different types of gene therapy. Give appropriate examples.
2. Explain the advantages and limitations of gene therapy approach.
3. Describe the molecular imaging. Explain different types of molecular imaging techniques.
4. What are the advantages and limitations of molecular imaging techniques?
5. Analyse the human genome project in detail. Write down the applications of HGP.

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UNIT 14 GENETIC ENGINEERING - TRANSGENICS

NOTES

Structure

14.0 INTRODUCTION

Genetic engineering or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup. Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. Transgenic organism are able to express the 'Trans' genes effectively due to the high similarity between the genetic sequences for proteins among different species.

Genetic engineering, also called genetic modification or genetic manipulation, is the direct manipulation of an organism's genes using biotechnology. It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms. New DNA is obtained by either isolating or copying the genetic material of interest using recombinant DNA methods or by artificially synthesising the DNA. A construct is usually created and used to insert this DNA into the host organism. The first recombinant DNA molecule was made by Paul Berg in 1972 by combining DNA from the monkey virus SV40 with the lambda virus. As well as inserting genes, the process can be used to remove, or "Knock Out", genes. The new DNA can be inserted randomly, or targeted to a specific part of the genome.

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Genetic engineering has been applied in numerous fields including research, medicine, industrial biotechnology and agriculture. In research GMOs are used to study gene function and expression through loss of function, gain of function, tracking and expression experiments. By knocking out genes responsible for certain conditions it is possible to create animal model organisms of human diseases. As well as producing hormones, vaccines and other drugs, genetic engineering has the potential to cure genetic diseases through gene therapy. The same techniques that are used to produce drugs can also have industrial applications such as producing enzymes for laundry detergent, cheeses and other products.

In this unit, you will study about the ethical issues in genetic engineering and transgenics, current developments, social, extrinsic, and intrinsic concerns, issue of species boundaries, and the legal implications of transgenics.

14.1 OBJECTIVES

After going through this unit, you will be able to:

- Understand the ethical issues in genetic engineering and transgenics
- Elaborate on the current developments
- Define the social, extrinsic, and intrinsic concerns
- Explain the issue of species boundaries
- Analyse the legal implications of transgenics

14.2 METHODOLOGY- TRANSGENICS

Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding. Hybrids refer to the transgenic organisms produced when germ cells (reproductive cells) from two different species combine to form an embryo which later develops into full-fledged organism. For instance, in nature, Mule is the best example observed in nature. It is produced by the copulation between a horse and a donkey. Further, chimeras are produced in labs by artificially combining genetic material from two different organisms into a single species.

The steps involved in creating a transgenic organism are as follows:

1. Identification of the Desirable Gene

The first step is the identification of the desirable gene or gene of interest that codes for a specific target protein. A gene that codes for a desirable trait or protein

must first be identified. Several molecular techniques like Gene Chips (Microarray) and DNA sequencing can be used to identify the desirable gene.

2. Isolation of Desirable Gene

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The second step involves the isolation of the desirable gene from the target species. It can be achieved either via mechanically breaking the cells or with the aid of chemical agents like detergents. The entire DNA can be then separated from the other cell components via technique known as cell centrifugation. Now, to separate the target gene from the total DNA content following steps needs to be followed:

- Separation of DNA fragments according to size via Gel Electrophoresis.
- Identification of the gene of interest using a DNA probe.
- Cut out of the gel and amplified (copied) using PCR.
- Alternatively, gene of interest could be inserted into a bacterial plasmid using the enzyme DNA Ligase.
- Bacteria would automatically copy the gene while undergoing cell division- a technique popularly called as Gene Cloning.
- However, if enough information is available regarding the gene of interest then it might be possible to create specific DNA primers and copy the gene using PCR without isolating it on a gel.

3. Transformation of the Desirable Gene

- Finally, a vector (varies according to cell type) is used to transfer the gene of interest into the organism being modified.
- The final DNA sequence that is prepared consisting of target gene and associated regulatory sequences (promoter and termination) sequences is referred to as Gene Construct.
- However, the success rate at which transgene is expressed is very low.
- For the target gene to be expressed, it must make its way into the nucleus.
- For it to be passed on during cell division (mitosis and meiosis) it must integrate itself into the target cells genome via recombination /crossing over.
- For verifying, whether, the target gene has been inserted into the genome or not- researchers incorporate a second gene known as reporter gene into the gene construct. This second gene codes for an easily selectable / observable characteristic like antibiotic resistance or glow in the dark protein.
- This enables the researchers to easily verify whether the integrated gene is expressing or not.

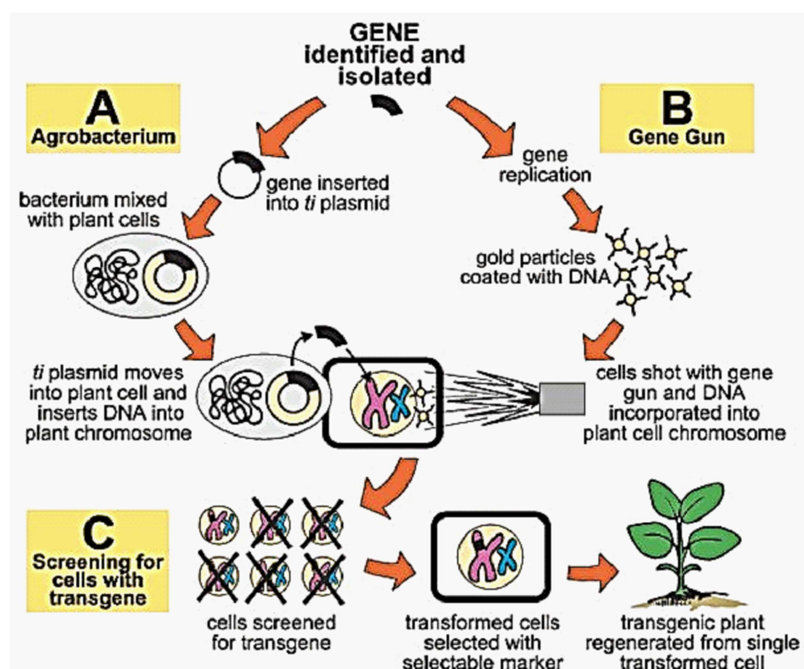


Fig. 14.1 *Methodology of Transformation of Gene*

14.3 CURRENT DEVELOPMENT IN THE FIELD OF TRANSGENIC

Genetic engineering, or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup. The field of transgenics enables scientists to create organisms that express a novel trait not found naturally in a species. In the next section, we will be discussing the current developments in the field of transgenics:

- The field of transgenics allows researchers to develop transgenic organisms that express a new improved trait which is generally absent in a species; for instance, to develop potatoes that are rich in protein content or rice that has elevated levels of vitamin A also referred to as golden rice.
- Research and development in the field of transgenics might help to save endangered species from deadly pathogenic species.
- Transgenic combinations might help researchers to develop vaccines against deadly diseases. For instance, the DNA of human tumour fragments is inserted into tobacco plants in order to develop a vaccine against non-Hodgkin's lymphoma.
- Likewise, researchers have produced a flu vaccine using human DNA and tobacco plants.

- Transgenic plants have been used to produce edible (safe to eat) vaccines for diseases like cholera, hepatitis B, rotavirus by incorporating a gene encoding human protein into fruits like tomatoes, and bananas, etc.
- A recent transgenic plant project referred as the "Glowing Plant Project", aims at incorporating a gene from a firefly into a houseplant, thus producing plants that exhibit a soft light in the darkness.
- Another proposal aims at producing plants that could illuminate streets and pathways hence saving the non-renewable energy sources
- Other potential industrial and medical applications comprise potent automotive and aerospace apparatus, powerful bioshields that can defend military personnel.
- Xenotransplantation, or the transplantation of living tissues or organs from one species to another has the potential approach to ease the scarcity of precious human organs like hearts and kidneys.
- Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.
- Researchers are also discovering the use of cell transplantation therapy for patients with critical disorders such as Parkinson's or spinal cord injury.
- Genetic manipulation of stem cells which involves the growth of tissues on scaffolding can be utilized as a provisional skin alternate for healing wounds or burns.
- Tissue engineering is rapidly becoming an appropriate substitute in methodology that involves replacement of human structure such as cerebrospinal shunts, heart valves, cartilage, and other organs.
- Industries are investing a lot of money in research and development to derive therapeutic proteins like monoclonal antibodies from the milk of transgenic animals such as rabbits, goats and cows to administer drugs in treatment protocols for disorders such as cancer, rheumatoid arthritis, and other autoimmune disorders.

To conclude, transgenics offer practical solution to patients as well as physicians for treating multiple disorders.

14.4 ETHICAL ISSUES

As studied in the above section, the field of transgenic biotechnology projects diverse possibilities in the positive way, however, these possibilities are not without possible risks. Some of the issues that need to be taken into consideration are as follows:

14.4.1 Social Concerns

- Provided the blending of animal and human DNA results in chimeric organisms having high degrees of intelligence never witnessed before in nonhuman animals, should these chimeric organisms be given exceptional rights as well as protection?
- What, if any of the social or legal controls or reviews should be placed on carrying out such research?
- What unintentional personal, social, and cultural consequences could result?
- One of the most important points for consideration is that who will have right to these technologies?
- How will be limited rather we can say inadequate resources like medical advances and novel treatments—be allocated to carry out such advance research?
- Various environmentalists, bioethicists and animal rights activists have argued that it is not ethically correct to generate animals that would go through pain as a consequence of genetic alteration. For instance- genetic alteration could lead to the development of a pig with no legs and hence such experimentation should be prohibited.

14.4.2 Extrinsic Concerns

- Risks and benefits associated with the use of experimental animals.
- What about the health risks associated with consumption of transgenics and genetically modified foods and crops?
- What if the GMOs have adverse effects on the health of human?
- The risk of creating novel diseases by combining different DNAs for which no treatment exist.
- What are the long-term effects on the natural environment when Genetically Modified Organism (GMOs) transgenic organisms are released?
- What if this result in the development of highly resistant weeds?
- What if it leads to development of antibiotic resistance by insect pests and other pathogens?
- What if it disturbs ecosystem in irreversible ways?
- What if it leads to development of highly pathogenic viruses and bacteria which might be dangerous to the existing crops?
- Should the research and development in the field of transgenics be limited to some extent and, if so, how should the limits decided? Also, it should be taken into consideration how to impose should the limits both nationally as well as internationally?

14.4.3 Intrinsic Concerns

- Are there any fundamental issues with creating new species in labs by using the methodology?
- Are species boundaries "Rigid" or 'Inflexible' or they should be viewed as a scale? What, if any, the outcomes of crossing the species boundaries?
- Are chimeras as well as transgenics organisms likely to suffer than "Traditional" organisms?
- Will transgenic interference in humans generate physical or behavioural traits which may or may not be easily distinguishable from what is typically supposed to be "Human"?
- What, if any, research taken in the field of genetic engineering is considered morally impermissible and banned- For instance, research and development in the military sector for destroying other nations.
- Will these interferences re-establish what it means to be "Normal"?

14.5 CONCEPT OF SPECIES BOUNDARY

Some individuals believe that crossing species boundaries is unnatural, immoral, and in violation of God's laws, which assumes that species boundaries are rigid and delineated. However, research conducted by researchers and published in several notable books and journal articles exhibit that the notion of fixed species boundaries is a hotly debated topic. Some bioethicist's points out that species concept is a broad concept and a variety of species concepts exist in the scientific literature such as: morphological, typological, biological, evolutionary, phylogenetic and ecological. All these species concepts reflect varying theories as well purpose for which researchers conceptualize and employ different species. However, it is said then if species boundaries are just a topic of a designation and there is no as such existence of true or fixed boundaries to cross, then several philosophical oppositions to transgenics organism will become less challenging.

As concluded in the above paragraph, the queries regarding the morality of transgenic animals is highly questionable yet numerous potential health risks might be associated with the transplantation of cells or organs between species. For instance, the transplantation of cells or organs between animal and human species might cause some fatal zoonotic disease. Following the rule of precautionary principle, i.e., better safe than sorry approach, it will be in the interest of general public to ban xenotransplantation trials using nonhuman primates until and unless the protocols have been marked sufficiently safe and ethical issues have been publicly addressed.

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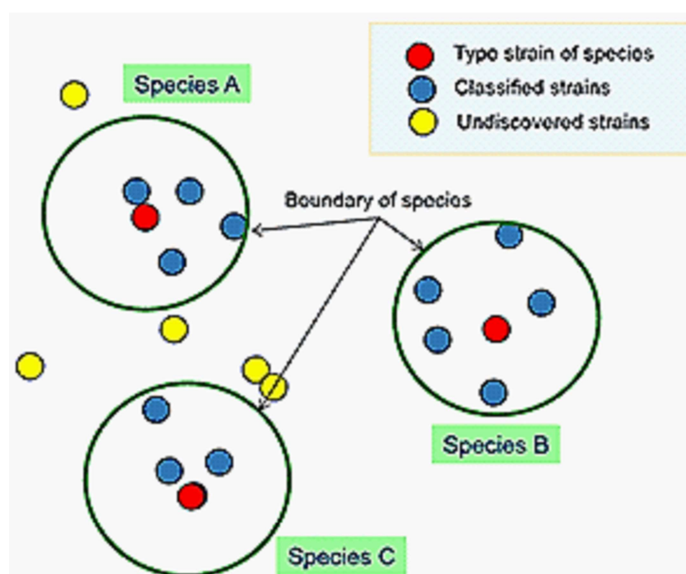


Fig. 14.2 *Concept of Species Boundary*

14.6 THE LEGAL IMPLICATIONS OF TRANSGENICS

There are several legal implications of transgenics as cited by numerous bioethicists from time to time. A few such legal issues will be highlighted in the following section:

- Several bioethicists have expressed their concern that such molecular technologies could be used to produce a race of subhumans that could be exploited.
- Delineation of species boundary is yet another concern.
- In future, if bioengineers succeed in creating a chimera between an animal and human, shall that creation be given all the rights as well as protection?
- Some bioethicists argue that the description of "Human Being" should be more open and defensive, rather than more limiting. However, others objects argue that more open or liberal descriptions can create a monetary hindrance to patenting creations that could be of possible use. This question needs legal addressing.
- Medical fraternity has expressed concern that athletes/celebrities might start using bioengineering to get an upper hand over their competitors.
- People with enormous wealth might be willing to genetically manipulate their children in order to make them successful in their respective fields or to have better looking kids or to make them better athletes/musicians/actors/ scientists or whatever else that might give them a benefit or upper hand.

- This practice might increase the ever-widening gap between common and rich people in the society. Also, this will close the door for less fortunate (in terms of money) to achieve something great even if they are talented.

NOTES

Check Your Progress

1. Define the term transgenic.
2. Interpret the methods of transgenic.
3. Elaborate on the xenotransplantation.
4. Why pig is used for carrying out the process of xenotransplantation?
5. Illustrate the concept of species.

14.7 ANSWERS TO CHECK YOUR PROGRESS QUESTIONS

1. Genetic engineering or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup. Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species. Transgenic organism are able to express the 'Trans' genes effectively due to the high similarity between the genetic sequences for proteins among different species.
2. (a) Identification of the desirable gene,

   (b) Isolation of the desirable gene, and

   (c) Transformation of the desirable gene.
3. Xenotransplantation is referred to as the transplantation of living tissues or organs from one species to another. It has the potential to ease the scarcity of precious human organs like hearts and kidneys.
4. Pigs have a comparable physiology and organ size to humans making them ideal organisms for transplantation into human recipients.
5. Species can be defined as populations that are diagnosable distinct, reproductively isolated, cohesive, or exclusive groups of organisms. Boundaries between species in sympathy are maintained by intrinsic barriers to gene exchange; these boundaries may not be uniform in space, in time, or across the genome.

14.8 SUMMARY

- Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species.

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- Hybrids refer to the transgenic organisms produced when germ cells (reproductive cells) from two different species combine to form an embryo which later develops into full-fledged organism.
- A transgenic organism is referred as one that contains a transgene introduced by technological methods (molecular tools and techniques used in laboratory) rather via the process of selective breeding. Hybrids refer to the transgenic organisms produced when germ cells (reproductive cells) from two different species combine to form an embryo which later develops into full-fledged organism.
- Genetic engineering, or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup.
- The field of transgenics allows researchers to develop transgenic organisms that express a new improved trait which is generally absent in a species; for instance, to develop potatoes that are rich in protein content or rice that has elevated levels of vitamin A also referred to as golden rice.
- Transgenic combinations might help researchers to develop vaccines against deadly diseases. For instance, the DNA of human tumour fragments is inserted into tobacco plants in order to develop a vaccine against non-Hodgkin's lymphoma.
- Some individuals believe that crossing species boundaries is unnatural, immoral, and in violation of God's laws, which assumes that species boundaries are rigid and delineated.
- Several bioethicists have expressed their concern that such molecular technologies could be used to produce a race of subhumans that could be exploited.

14.9 KEY WORDS

- **Transgenics:** Transgenics refers to the molecular technique which involves removal of genetic material from one species of animal or plant and adding it to altogether different species.
- **Genetic engineering:** Genetic engineering, or genetic modification is a field of biotechnology and bioengineering which aims at uses of multiple tools and techniques to modify an organism's genetic makeup.
- **Tissue engineering:** Tissue engineering is rapidly becoming an appropriate substitute in methodology that involves replacement of human structure such as cerebrospinal shunts, heart valves, cartilage, and other organs.

- **Concept of species boundary:** Some individuals believe that crossing species boundaries is unnatural, immoral, and in violation of God's laws, which assumes that species boundaries are rigid and delineated.
- **Legal implications of transgenics:** Several bioethicists have expressed their concern that such molecular technologies could be used to produce a race of subhumans that could be exploited.

14.10 SELF ASSESSMENT QUESTIONS AND EXERCISES

Short-Answer Questions

1. Explain the term transgenic.
2. Illustrate the methods of transgenic.
3. Define the xenotransplantation.
4. Why pig is used for carrying out the process of xenotransplantation?
5. State the concept of species.

Long-Answer Questions

1. Discuss in detail the methodology of transforming desirable gene in target organism.
2. Write down the applications of transgenic organism.
3. Describe the concept of species boundary with the help of examples.
4. What are the ethical concerns of methodology of transgenics?
5. Explain the legal implications associated with the transgenics.

14.11 FURTHER READINGS

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